thio-β-D-glucopyranoside[20] in 125 ml of EtOH was stirred at reflux temp for 1.5 hr. Afterwards, the mixt was concd under reduced pressure and the residue partitioned between H_2O and $CHCl_3$. The organic exts were dried (Na_2SO_4), filtered, and concd *in vacuo*, and the residue was recrystd from MeOH to give 14.2 g (87%) of solid: mp 110–112°; [α]D ($CHCl_3$) −33.2° (*c* 1.246). *Anal.* ($C_{18}H_{26}O_{10}S_2$) C, H, S.

2-Acetylthioethyl Tetra-*O*-acetyl-β-D-glucopyranoside (15). An EtOH soln of 5.85 g (0.014 mole) of 2-chloroethyl tetra-*O*-acetyl-β-D-glucopyranoside[21] was allowed to react with KSAc as in 14. The residue was recrystd from MeOH to give 4.6 g (72%): mp 80–81°; [α]D ($CHCl_3$) −3.8° (*c* 1.016). *Anal.* ($C_{18}H_{26}O_{11}S$) C, H, S.

Antiarthritic Assay Method.[14] Adjuvant arthritis was produced by a single intradermal injection of 0.75 mg of *Mycobacterium butyricum* suspended in white paraffin oil (light N.F.) into the left hind-paw footpad. The injected leg becomes inflamed (increased vol) and reaches max size within 3–5 days (primary lesion). The animals exhibit a decrease in body wt gain during this initial period. Adjuvant arthritis (secondary lesion) occurs after a delay of approx 10 days and is characterized by inflammation of the noninjected sites (right hind leg), decrease in body wt gain, and further increases in the vol of the injected hind leg. Test compds were administered daily, beginning on the day of adjuvant injection for 17 days exclusive of days 4, 5, 11, and 12. Drug activity on the primary (left leg day 3) and secondary (both legs day 16) lesions was detd by comparing leg vols of the treated group with a control arthritis (vehicle) group. Hind-leg vols were measured by immersing the leg into a Hg reservoir and recording the subsequent Hg displacement. A compd was considered to have antiarthritic activity if it produced a statistically significant ($p < 0.05$) decrease in the inflamed hind-leg volumes when compared with arthritic controls. The level of significant difference between treated groups and control groups was determined by the Student's *t* test.

Acute Single Dose Lethality. Male Charles River rats weighing between 160 and 220 g were divided randomly into groups of ten rats each. The groups were treated orally with test compd. After dosing, all animals were obsd for acute deaths on day 1 and delayed deaths over a 10-day period. All LD_{50}'s and their confidence limits were calcd using the Litchfield-Wilcoxon method.[22]

Acknowledgments. We are indebted to Miss Margaret Carroll, Mr. Walter Hamill, and their associates for analytical and physical data and Mr. Edward Macko for toxicity data. We thank Dr. Carl Kaiser, Dr. Joseph Weinstock, and Miss Helen Ebert for helpful suggestions.

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New Substrates for a Pancreatic Exocrine Function Test

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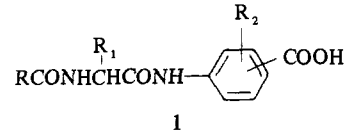
Three-unit compounds, such as 4-(*N*-benzoyl-L-tyrosyl)aminobenzoic acid which, in general, comprise a sensitive peptide linkage to tracer aminobenzoic acids, were prepared and shown in rat tests to possess considerable discrimination for *in vivo* chymotrypsin activity when ingested. They may, therefore, be useful in the clinical detection of pancreatic exocrine insufficiency. Thirteen of these compounds are listed in Table I. The peptide linkage was formed by the unique reaction of mixed anhydride of the appropriate acylated α-amino acid with the free aminobenzoic acid, with toluenesulfonic acid catalyst. *In vitro* half-time values were determined for the hydrolysis of these compounds in the presence of chymotrypsin and are compared with *in vivo* data. Ortho substitution in the aminobenzoic acid portion strongly affected both optical activity and hydrolysis rate.

Reliable clinical measurement of pancreatic exocrine function must be carried out on duodenal aspirates and is therefore difficult to conduct on a routine basis. As part of an attempt to devise a simpler test, we have prepared new compounds containing a peptide bond, which, when ingested, would be split specifically by the pancreatic enzyme, chymotrypsin, to yield a nontoxic tracer which could be recovered in the urine. This paper reports the chemistry, some *in vitro* rate data, and correlation of the latter with *in vivo* data on rats. Tests in three animal species have been reported for compound **1**.[1]



1

We selected *p*-aminobenzoic acid (PABA) and, more generally, the aminobenzoic acids (ABA's) as the tracers, be-

cause, when taken by mouth, they are rapidly and well absorbed, are not appreciably metabolized, and are recovered in good yield in the urine.[2] Although more complicated structures incorporating PABA were useful, only three elements of structure were actually needed. These elements were incorporated in the compounds described here, which comprise a central amino acid residue attached through its carboxyl group to the ABA and through its amino group to a protective acyl group, RCO.

The central amino acid residue was derived from those amino acids whose carboxylic peptide links are typically hydrolyzed in the presence of chymotrypsin, *i.e.*, the L forms of tyrosine, phenylalanine, and tryptophan, and, much less effectively, L-methionine and L-leucine. Variations were made in the acyl group, which serves to prevent cleavage by aminopeptidase. The ABA's, being unnatural amino acids, do not offer a site for carboxypeptidase, which is also present in the intestine. Ring substitution and position isomerism were varied in the ABA residue.

This report describes only the free acids, which were more effective than other derivatives in the test animal. Acid derivatives (esters, amides, etc.) were quite insoluble at intestinal pH, but the acids did have a little solubility, the degree of which was undoubtedly an important part of *in vivo* effectiveness, as will be developed later.

The mixed carbonic anhydride method[3] was used to prepare the compounds from the appropriate acylamino acids. Fortunately, we found that in the presence of a small amount of strong acid, such as *p*-toluenesulfonic acid, the ABA's would undergo direct reaction with the mixed anhydride. The facility of this unique reaction, which takes place in the face of an increasing ratio of carboxyl group to available amine, is perhaps owing to interchange of the strong acid (HX) with the mixed anhydride to give low concentrations of very active acylating agent.

$$RCONHCH(R_1)COOCOOEt + HX \rightarrow RCONHCH(R_1)COX + EtOH + CO_2$$

$$RCONHCH(R_1)COX + NH_2\text{-}C_6H_3(R_2)\text{-}COOH \rightarrow 1 + HX$$

The products are listed in Table I. Some racemization was unavoidable, and complete separation of racemate from L isomer was not attempted. The optical purity, in terms of available L isomer, is given by the degree of hydrolytic cleavage to ABA over a 24-hr period at a high molar ratio, normally 1:700, of chymotrypsin to compound (Table I). The value for compound **4**, which is racemic, confirms the relative accuracy of this method. Compound **6**, from L-leucine, was too slowly hydrolyzed for this determination, but the value for the optical rotation was in line with those of compounds which contained very little racemate.

The compounds were hydrolyzed under standard conditions in Tris buffer with standard chymotrypsin, as detailed in the Experimental Section. Half-times were estimated from a plot of the data. The compounds were also given, at a level of 10 mg of ABA per kilogram body weight, to rats having a ligated and sectioned common bile duct (CBDL rats) and to sham-operated rats. Urine was collected from these rats for a period of 6 hr, and its ABA content was determined after acid hydrolysis to convert ABA metabolites to the parent compound.[4] Table II presents the results of these two sets of experiments (a more detailed account of *in vivo* tests will be published elsewhere).

The three sets of data provide (1) a comparison of the effectiveness with which a given compound is hydrolyzed by chymotrypsin *in vitro* and *in vivo* (sham-operated rats), and (2) a measure of the *in vivo* specificity of the compounds toward chymotrypsin. The common bile duct ligation excludes only the pancreatic enzymes, while all the other proteolytic enzymes normal to the GI tract can exert their catalytic potential. The CBDL data indicate that these enzymes promoted some hydrolysis, but the recovery of ABA's in the CBDL rats was usually one-third or less of the recovery of ABA's in the sham rats, thus confirming the essential requirement for chymotrypsin under these conditions.

Good *in vivo* recoveries, in some cases approaching the total recovery of the ABA's when fed alone, were obtained from compounds which were relatively rapidly hydrolyzed *in vitro*, as with **1**, **2**, **3**, **8**, **10**, **11**, **12**, and **13**. There was not, however, as close a correspondence as might be anticipated. Benzoyltyrosyl-, -tryptophyl-, and -phenylalanyl-*p*-aminobenzoic acids (**1**, **2**, and **3**) were many orders of magnitude more sensitive than the corresponding derivatives of methionine and leucine (**5** and **6**) in *in vitro* studies. There was less difference in the animal. The aliphatic acyl derivatives **10**, **11**, and **12** were more slowly hydrolyzed *in vitro*

Table I. Test Compounds

$$RCONHCH(R_1)CONH\text{-}C_6H_2(R_2)(R_3)\text{-}R_4$$

Compd	R	NHC(R_1)HCO	R_2	R_3	R_4	Mp, °C	Crystallized from[a]	$[\alpha]^{25}D$[b]	Equilibrium cleavage, %	Formula[c]
1	C_6H_5	L-Tyr	H	H	COOH	243-244	A	+87	96	$C_{23}H_{20}N_2O_5$
2	C_6H_5	L-Trp	H	H	COOH	170-174	B	+76	85	$C_{25}H_{21}N_3O_4$
3	C_6H_5	L-Phe	H	H	COOH	245-252	A	+79	88	$C_{23}H_{20}N_2O_4$
4	C_6H_5	DL-Phe	H	H	COOH	248-252	A	0	53	$C_{23}H_{20}N_2O_4$
5	C_6H_5	L-Met	H	H	COOH	203-204	C	+66	100[d]	$C_{19}H_{20}N_2O_4S$[e]
6	C_6H_5	L-Leu	H	H	COOH	198-200	C	+95	*f*	$C_{20}H_{22}N_2O_4$
7	C_6H_5	L-Tyr	COOH	H	H	204-208	A	+2	88[d]	$C_{23}H_{20}N_2O_5$
8	C_6H_5	L-Tyr	H	COOH	H	249-253	A	+57	95	$C_{23}H_{20}N_2O_5$
9	C_6H_5	L-Tyr	CH_3	H	COOH	242-244	A	−3	97[d]	$C_{24}H_{22}N_2O_5$
10	CH_3	L-Tyr	H	H	COOH	229-231	B	+92	90	$C_{18}H_{18}N_2O_5$
11	C_2H_5	L-Tyr	H	H	COOH	241-242	B	+90	92	$C_{19}H_{20}N_2O_5$
12	n-C_3H_7	L-Tyr	H	H	COOH	223-226	B	+78	90	$C_{20}H_{22}N_2O_5$
13	C_2H_5O	L-Tyr	H	H	COOH	136-138	C	+84	90	$C_{19}H_{20}N_2O_6$[g]

[a] Recrystallizing solvents: A, methanol-water; B, ethyl acetate-petroleum ether; C, ethyl acetate. [b] 1% in dimethylformamide. [c] Analyzed for C, H, and N. [d] Measured at 10 times usual chymotrypsin concentration. [e] S: calcd, 8.6; found, 8.7. [f] Too slow to determine at 10× concentration. [g] C: calcd, 61.3; found, 61.8.

Table II. Urinary Excretion of ABA's by Rats Following Oral Administration of ABA-Containing Compounds

Compd administered	*In vitro* half-time (hr)	Average ABA recovery (%) Sham	CBDL
1	1.5	57	9
2	1.3	33	13
3	2.3	75	27
4	1.5[a]	9	2
5	24	22	8
6	24	16	8
7	>24[b]	7	2
8	1.3	43	2
9	>24[c]	18	1
10	24	78	23
11	10	69	29
12	3.5	67	11
13	16	65	7
PABA		73	75
OABA[d]		60	

[a] L isomer only. [b] 25% in 24 hr. [c] 32% in 24 hr. [d] Anthranilic acid.

than the benzoyl derivatives and showed an interesting progression. These differences again were not seen *in vivo*.

The leavening factor may well be that of relative concentration in the actual *in vivo* situation. For example, the carbamate **13** and the acyl derivatives **10**, **11**, and **12** might be expected to have higher solubility in intestinal fluid than the benzoyl derivative **1**.

Strong ortho interaction was observed in our work, qualitatively similar to the effect of *o*-nitro groups in phenyl esters[5] and anilides.[6] Compounds **7** and **9** were hydrolyzed only slowly *in vitro* and in the animal. Startling differences in optical rotation of these compounds as compared with other substrates (Table I), differences which are independent of the electronic character of the ortho substituent of the ABA, suggest that this interaction is primarily steric in nature, increasing the comparative value of the Michaelis constant, K_m, and reducing the velocity of the hydrolytic reaction. The meta isomer **8** was essentially equivalent to the para isomer **1** indicating that, under the conditions, the carboxyl group did not exert a strong electronic effect.[5]

Finally, it will be seen from the racemic compound **4**, Tables I and II, that the L form was completely hydrolyzable without competitive inhibition from the D form *in vitro*. The expected distinction between sham-operated and CBDL rats was observed, but PABA recovery was less than the expected one-half of that from the L isomer.

The *in vivo* results are encouraging in the sense that the specificity toward the pancreatic enzyme seems to be largely maintained. Other enzymes, such as pepsin, carboxypeptidase, and other di- and tripeptidases, which are present in the gastrointestinal tract, seem to act only very slowly, if at all, on the compounds. On the whole, the results support the general applicability of the concepts and provide hope for a practical solution to the problem of a simple pancreatic function test.

Experimental Section

Determination of *in Vitro* Half-Times. The compound was dissolved in Tris buffer, adjusted to pH 7.8, to give a 5×10^{-4} *M* solution. A stock solution of enzyme (Sigma Chemical Co., γ-chymotrypsin, bovine pancreas twice crystallized and lyophilized, salt-free Type II) was made at a 1 mg/ml concentration in 0.001 *N* hydrochloric acid. Hydrolysis was begun by combining 29 ml of substrate solution with 0.5 ml of 0.001 *N* hydrochloric acid and 0.5 ml of chymotrypsin solution. Ambient temperature (25°) was used. Samples were withdrawn at appropriate intervals and analyzed by the Bratton Marshall technique,[7] with the appropriate ABA used in the blank. Half times are estimated from a plot of the data.

Preparative Details. Benzoyl-L-tyrosine,[8] benzoyl-L-tryptophan,[9] benzoyl-L-phenylalanine,[10] benzoyl-L-methionine,[11] and benzoyl-L-leucine[12] were prepared by known methods. The condensation of benzoyl-L-tyrosine with PABA illustrates the mixed anhydride method used in the preparations.

4-(*N*-Benzoyl-L-tyrosyl)aminobenzoic Acid (1). Benzoyl-L-tyrosine (28.5 g, 0.1 mole) was dissolved in tetrahydrofuran (300 ml), the solution was cooled to −15°, and *N*-methylmorpholine (11 ml, 0.1 mole) was added. Ethyl chloroformate (10 ml, 0.1 mole) was added at −10 to −15°. After 15 min, a solution of *p*-aminobenzoic acid (13.7 g, 0.1 mole) in tetrahydrofuran (125 ml) was added, along with a solution of *p*-toluenesulfonic acid (1.9 g, 0.01 mole) in tetrahydrofuran, again at −10 to −15°. The temperature was allowed to rise to 5°, and the mixture was maintained there for 2.5 hr. It was then poured into cold 0.1 *N* hydrochloric acid (5 l.), filtered, washed, and dried to yield 42 g of almost pure product, mp 221–225°, $[\alpha]^{25}D$ +77°. The product was recrystallized by dissolving it in a mixture of methanol (450 ml) and water (200 ml), adding 100 ml of water, and cooling to −20°. The precipitated product weighed 29.5 g (73%).

Acetyl, propionyl, butyryl, and ethoxycarbonyl derivatives of L-tyrosyl-PABA were prepared directly from the acid chlorides, L-tyrosine, and PABA without isolating the intermediate acyltyrosine, as illustrated below.

4-(*N*-Propionyl-L-tyrosyl)aminobenzoic Acid (11). To a slurry of L-tyrosine (72.4 g, 0.4 mole) in tetrahydrofuran (500 ml) was added propionyl chloride (17.2 ml, 0.2 mole). The mixture was stirred overnight at room temperature, and the precipitated L-tyrosine hydrochloride (43.5 g, 100%) was then removed by filtration. The filtrate was cooled to −15° and to it were added *N*-methylmorpholine (22 ml, 0.2 mole) and ethyl chloroformate (20 ml, 0.2 mole) at −10 to −15°. After 15 min, there were added *p*-aminobenzoic acid (27.4 g, 0.2 mole) and *p*-toluenesulfonic acid (3.8 g, 0.02 mole). After 0.5 hr at −15° and 3 hr at 5°, the mixture was poured into cold 0.1 *N* hydrochloric acid, as above, to give 46 g (65%) of crude product. The product was recrystallized first from ethanol, ethyl acetate, and petroleum ether and then from methanol and water to give 36 g (51%) of product.

Acknowledgment. Dr. Norton J. Greenberger suggested the original idea for this work.

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