Preparation and Application of the 5-(4-(9-Fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)-valeric Acid (PAL) Handle for the Solid-Phase Synthesis of C-Terminal Peptide Amides under Mild Conditions[1-3]

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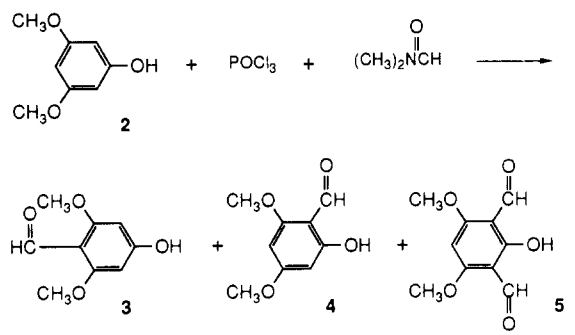
The acid-labile 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle 1 is described for the solid-phase synthesis of C-terminal peptide amides. The pure para isomer of 1 was prepared by each of two efficient five-step routes, in overall yields from 52% to 74%. The handle 1 was coupled onto a variety of amino group-containing supports to provide a general starting point for stepwise assembly of peptide chains according to a wide range of chemistries. In particular, protocols based on the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) group worked well with PAL handle 1. For small model peptides, final cleavage of *tert*-butyl side-chain protecting groups and of the anchoring linkage proceeded smoothly in trifluoroacetic acid–dichloromethane–dimethyl sulfide (14:5:1) (reagent A) at 25 °C for 2 h. For cleavage of complex peptides that contain several sensitive side-chain functionalities, or that include arginine residues blocked with the 4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr) or 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) groups, a mixture of trifluoroacetic acid–thioanisole–1,2-ethanedithiol–anisole (90:5:3:2) (reagent R), applied for 2–8 h at 25 °C, was preferred. A side reaction involving alkylation at tryptophan was elucidated, and conditions were developed to minimize its occurrence. The methodology was demonstrated by syntheses of over a hundred peptides, among which acyl carrier protein (65–74) amide (natural and retro sequences), luteinizing hormone-releasing hormone, adipokinetic hormone, PHI porcine fragment (18–27), and human gastrin-I are highlighted in this report. In comparative studies, the yields and purities of peptide amides prepared with PAL were shown to be equivalent or superior to those found for products prepared by alternative procedures from the recent literature.

One important aspect to achieving milder chemical methods for solid-phase peptide synthesis[6] is to define the mode of attachment (*anchoring*) of the terminal residue to a polymeric support. We have developed a number of *handles*,[2a-c,7] which are compatible with readily removable

Scheme I



(1) Abbreviations used are as follows: AA, amino acid residue (free or protected, depending on context); BHA, benzhydrylamine (resin); Boc, *tert*-butyloxycarbonyl; BOP, benzotriazolyl *N*-oxytris(dimethylamino)-phosphonium hexafluorophosphate; Bpoc, 2-(*p*-biphenylyl)prop-2-yloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; Dts, dithiasuccinoyl; EDT, 1,2-ethanedithiol; EtOAc, ethyl acetate; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; LH-RH, luteinizing hormone-releasing hormone; Linker-AM, 2-(4-Fmoc-aminomethyl(2,4-dimethoxyphenyl)-phenoxy)acetic acid; MBHA, *p*-methylbenzhydrylamine (resin); MPLC, medium-pressure liquid chromatography; Mtr, 4-methoxy-2,3,6-trimethylphenylsulfonyl; NMM, *N*-methylmorpholine; Npys, 3-nitro-2-pyridinylsulfenyl; OSu, *N*-succinimidoyl ester; PAL, title handle of this paper (ref 8); Pepsyn K, kieselguhr-encapsulated polydimethylacrylamide; Pmc, 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl; PHI, porcine intestinal peptide as described by Tatemoto and Mutt (ref 31) with N-terminal histidine and C-terminal isoleucine; Ⓐ, resin support; reagent A, trifluoroacetic acid–dichloromethane–dimethyl sulfide (14:5:1); reagent R, trifluoroacetic acid–thioanisole–1,2-ethanedithiol–anisole (90:5:3:2); TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; Trt, triphenylmethyl (trityl).

(2) Preliminary reports: (a) Albericio, F.; Słomczyńska, U.; Barany, G. In *Forum Peptides Le Cap d'Agde 1984*; Castro, B., Martinez, J., Eds.; Les Impressions Dohr; Nancy, France, 1986; pp 1–5. (b) Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1987**, *30*, 206–216. (c) Albericio, F.; Kneib-Cordonier, N.; Gera, L.; Hammer, R. P.; Hudson, D.; Barany, G. In *Peptides—Chemistry and Biology, Proceedings of the Tenth American Peptide Symposium*; Marshall, G. R., Ed.; Escom Science Publishers: Leiden, The Netherlands, 1988; pp 159–161. (d) Biancalana, S.; Hudson, D.; Frankel, A. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 7397–7401.

(3) Taken in part from the Ph.D. Thesis of N. Kneib-Cordonier, University of Minnesota, 1989.

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(6) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154. (b) Review: Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1–284. (c) Merrifield, R. B. *Science* **1986**, *232*, 341–347. This is an updated version of Merrifield's Nobel Prize lecture, which has been reprinted in several other journals. (d) Review: Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. In *Encyclopedia of Polymer Science and Engineering*; Kroschwitz, J. E., Ed.; Wiley: New York, 1988; Vol. 12, pp 811–858. (e) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Peptide Protein Res.* **1987**, *30*, 705–739. In spite of the relative publication years, ref 6e represents a somewhat condensed and updated version of ref 6d.

(7) *Handles* are defined as bifunctional spacers which serve to attach the initial residue to the polymeric support in two discrete steps. One end of the handle incorporates features of a smoothly cleavable protecting group, and the other end allows facile coupling to a previously functionalized support. For prior work from this laboratory on handles, see: (a) Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1984**, *23*, 342–349. (b) Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1985**, *26*, 92–97. (c) Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942. (d) Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1987**, *30*, 177–205. (e) Mullen, D. G.; Barany, G. *J. Org. Chem.* **1988**, *53*, 5240–5248 and references cited in all of these papers.

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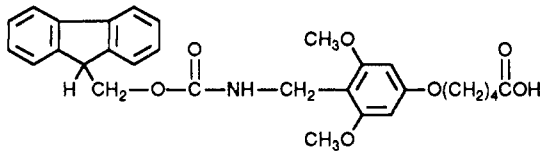
N^{α}-amino protecting groups such as the highly acid-labile 2-(*p*-biphenylyl)prop-2-yloxycarbonyl (Bpoc), the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc), or the thiolyzable N^{α}-dithiasuccinoyl (Dts) or N^{α}-3-nitro-2-pyridinylsulfenyl (Npys) functions. The present paper focuses on one such handle, namely 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid (1) (PAL),[2,8,9] which furnishes a tris(alkoxy)benzylamide anchoring linkage[2] that cleaves upon mild acidolysis to provide C-terminal peptide amides. Handle 1 fills a significant niche in the arsenal of tools for peptide synthesis, since a number of naturally occurring peptides, including oxytocin, secretin, apamin, calcitonin, thymosin, and several releasing hormones from the brain, are isolated as C-terminal peptide amides. Of further biological significance, since peptide acids are ionized at physiological pH, partial sequences of proteins required for immunological studies are probably best prepared as their amides. Alternative approaches to peptide amides have been reported from other laboratories within the past few years.[10,11]



"PAL" 1

We present here the details of improved procedures for the preparation of 1, which have been carried out on larger scales to provide a commercial product.[8] The application of 1 to peptide synthesis is described, including results with the very challenging 17-residue peptide amide human gastrin-I.[12,13] A significant consideration for optimizing yields is the nature and concentration of scavengers added to the cleavage cocktail, and this issue has been explored systematically.

Results

Regioselective Routes to PAL Handle. Our initial reports[2a,b] detailed the systematic screening of a series of alkoxy-substituted benzylamides and concluded that handle 1 was best suited for application to the preparation of C-terminal peptide amides. For that work, the desired compound was made in seven steps from 3,5-dimethoxyphenol (2). The overall yield was 15%, and a mixture of positional isomers was obtained.[9] We now describe improvements in reaction conditions at several points with a concomitant decrease in the number of steps. Furthermore, pure positional isomers can be obtained by changing the order of steps. These advances are primarily of interest for the chemistry, since anchoring linkages derived from the pure para isomer 1 do not undergo acidolysis at significantly better rates than those from the pure ortho isomer 1′ (data in supplementary material).

The new routes start with the isomeric hydroxybenzaldehydes 3 and 4, obtained by Vilsmeier formylation[14] of 2 (Scheme I). This reaction, as improved in our hands, gave 3 and 4 in about equal levels, as well as a small amount of the diformyl species 5. The solubility properties of 3,[14] 4,[15] and 5[16] are quite different, and each of these compounds was readily obtained in high purity. The phenolic functions of 3 or 4 were each alkylated with ethyl 5-bromovalerate (6), under improved conditions,[17] to provide 7 or 7′, respectively (Scheme II, left side). The ethyl valerate intermediates 7 could either be isolated or

(8) Since 1988, the title handle and closely allied structures have been made commercially in >100-g lots and marketed by MilliGen/Biosearch under the user-friendly acronym PAL (Peptide Amide Linker). The corresponding functionalized support is marketed as PAL-Resin.

(9) Handle 1 is depicted as the isomer in which the aminomethyl function is para to the valeryl linking group. The corresponding ortho isomer is designated as 1′. In our earlier work (ref 2b), the PAL handle was obtained as a 1:2 mixture of 1:1′. With the optimal acid/scavenger cleavage combinations reported in the text, Boc-amino acyl PAL-resins provide the appropriate *pure* amino acylamide in nearly quantitative yields, irrespective of the anchored residue. Supplementary Table I shows that with less effective reagents, e.g., CF_3COOH–CH_2Cl_2 (1:1, v/v) or 3.5 N HCl in dioxane, 1 h, 25 °C, cleavage yields starting with para isomer 1 were only marginally higher than those starting with ortho isomer 1′. Most significantly, the yield for the known difficult acidolysis at phenylalanine was found to be only slightly less than that for the straightforward acidolysis at glycine.

(10) For recent handles (or resin supports) directed toward peptide amides, see: (a) Rich, D. H.; Gurwara, S. K. *Tetrahedron Lett.* **1975**, 301–304. (b) Colombo, R. *J. Chem. Soc., Chem. Commun.* **1981**, 1012–1013. (c) Penke, B.; Rivier, J. *J. Org. Chem.* **1987**, *52*, 1197–1200. (d) DeGrado, W. F.; Wolfe, H. R. (DuPont, Inc., Wilmington, DE), unpublished observations and personal communications, 1987–1988. (e) Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 2107–2110. (f) Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787–3790. (g) Breipohl, G.; Knolle, J.; Stüber, W. *Tetrahedron Lett.* **1987**, *28*, 5651–5654. (h) Funakoshi, S.; Murayama, E.; Guo, L.; Fujii, N.; Yajima, H. *Collect. Czech. Chem. Commun.* **1988**, *53*, 2791–2800. (i) Penke, B.; Nyerges, L.; Klenk, N.; Nagy, K.; Asztalos, A. In *Peptides, Chemistry, Biology, Interactions with Proteins*; Penke, B., Tórök, A., Eds.; Walter der Gruyter: Berlin, 1988; pp 121–126. (j) Penke, B.; Nyerges, L. In *Peptides 1988: Proceedings of the 20th European Peptide Symposium*; Jung, G., Bayer, E., Eds.; Walter der Gruyter: Berlin, 1989; pp 142–144. (k) Stüber, W.; Knolle, J.; Breipohl, G. *Int. J. Peptide Protein Res.* **1989**, *34*, 215–221. (l) Hammer, R. P.; Albericio, F.; Gera, L.; Barany, G. *Int. J. Peptide Protein Res.*, in press. This listing, while not exhaustive, highlights the most significant approaches.

(11) In addition to the MilliGen/Biosearch product PAL (ref 8), commercial products are available through Bachem, Calbiochem, Cambridge Research, DuPont, and Novabiochem, based on the chemistries of Penke and Rivier (ref 10c), Rink (10f), and Breipohl et al. (refs 10g,k). Tradenames include Linker-AM and RapidAmide resin.

(12) Gastrointestinal peptides have been reviewed: Bodanszky, M.; Kwei, J. Z. *Gastrointestinal Hormones*; Glass, G. B., Ed.; Raven Press: New York, 1980; pp 413–432. Isolation and sequencing of human gastrin is described by: Gregory, R. A.; Tracy, H. J. *Nature* **1966**, *209*, 583, Bentley, P. H.; Kenner, G. W.; Sheppard, R. C. *Nature* **1966**, *209*, 583–585.

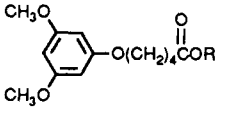
(13) For prior syntheses of sequences in the gastrin family, both in solution and by solid-phase, see: (a) Beacham, J.; Bentley, P. H.; Gregory, R. A.; Kenner, G. W.; MacLeod, J. K.; Sheppard, R. C. *Nature* **1966**, *209*, 585–586. (b) Jaeger, E.; Thamm, P.; Schmidt, I.; Knof, S.; Moroder, L.; Wünsch, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 155–164. (c) Fries, L.; Coy, D. H.; Huang, W. Y.; Meyers, C. A. In *Peptides: Structure and Biological Function. Proceedings of the Sixth American Peptide Symposium*; Gross, E., Meienhofer, J., Eds.; Pierce Chemical Co.: Rockford, IL, 1979; pp 499–502. (d) Brown, E.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1161–1167. (e) Tam, J. P.; Merrifield, R. B. *Int. J. Peptide Protein Res.* **1985**, *26*, 262–273 and references cited therein.

(14) (a) Gruber, W. *Chem. Ber.* **1943**, *76*, 135–142. (b) Jain, A. C.; Lal, P.; Seshadri, T. R. *Indian J. Chem.* **1968**, *6*, 485–487. (c) Carvalho, C. F.; Sargent, M. V. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1605–1612.

(15) (a) Herzig, J.; Wenzel, F.; Kerenyi, E.; Gehringer, H. *Monatsch. Chem.* **1903**, *24*, 857–880. (b) Pratt, D. G.; Robinson, R. *J. Chem. Soc.* **1924**, *25*, 188–199. (c) This compound can be obtained from Aldrich Chemical Co.

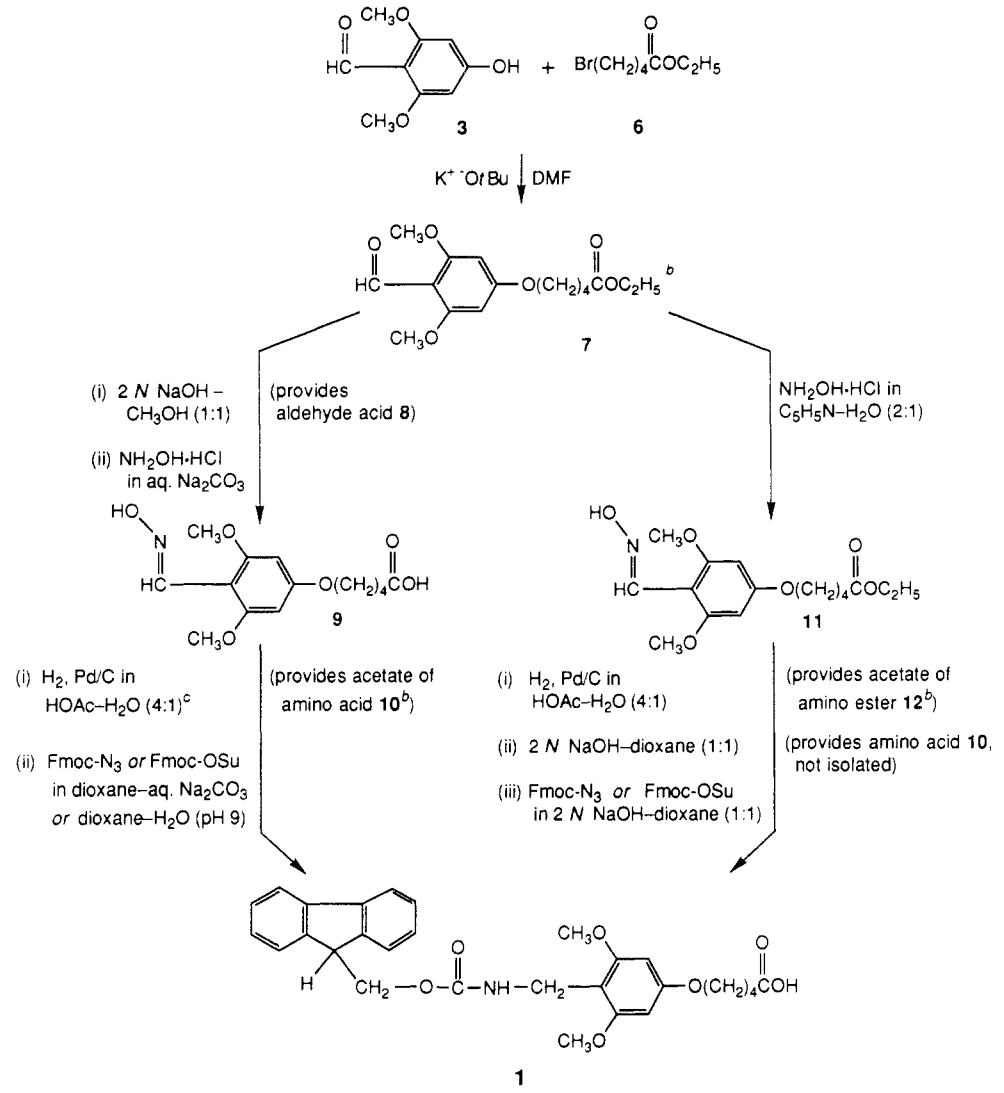
(16) Gruber, W.; Traub, F. *Monatsch. Chem.* **1947**, *77*, 414–430.

(17) In ref 2b, alkylation of 2 with 6 was promoted by lithium hydride in DMF under forcing conditions, giving ethyl 5-(3,5-dimethoxyphenoxy)valerate (14) in modest yield. In that work, 14 was not isolated, but carried forward two more steps to provide an intermediate suitable for analysis. Our current improved alkylation conditions also permit isolation of analytically pure 14 in excellent yield (see the Experimental Section). We also describe saponification of 14 to acid 15, which is formed in only low yield by direct alkylation of 2 with 5-bromovaleric acid.



14: R = C_2H_5
15: R = H

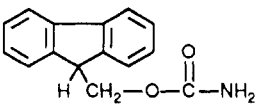
Scheme II[a]



[a] The para isomers are drawn. For the left pathway, corresponding reactions were carried out starting with 2-formyl-3,5-dimethoxyphenol (4) to give all of the corresponding ortho isomers. In the Experimental Section, the ortho isomers are given the same structural formula number, with a prime (′) added. [b] This intermediate can be used directly in the next step without purification. [c] These conditions avoid a re-esterification problem of an earlier route (ref 2b).

saponified directly to the corresponding acids **8**. The pure isomers **8** (earlier obtained[2b] as the mixture of isomers) were each smoothly converted to oximes **9**, which were then reduced to amino acids **10** and then treated directly with appropriate reagents to introduce Fmoc protection (Scheme II). Overall, crystalline para handle **1** was obtained in five steps and 52% yield from **3**, whereas crystalline ortho handle **1′** was obtained in 25% yield from **4**.

An alternative route (Scheme II, right side) transformed[18] aldehyde ester **7** to the oxime ester **11**, which was then reduced to amine **12**, saponified (without isolation) to **10**, and converted to **1**. The overall yield was 74% for five steps based on **3**. Some ammonia formed during the large-scale reduction of **11** carried over, with 9-fluorenylmethyl carbamate ($FmocNH_2$) (**13**)[19] being produced during the final derivatization step. Compound **13**, which in any event cannot interfere with the ultimate application of handle **1**, remains in the mother liquor when **1** is recrystallized from ethyl acetate.



13

Use of the PAL Handle in Solid-Phase Peptide Synthesis. Handle derivative **1** was coupled onto a series of amino group containing supports by a variety of procedures. Starting with polystyrenes functionalized with aminomethyl, amino acyl, or *p*-methylbenzhydrylamine (MBHA) groups, handle **1** (1.2–2.0 equiv) was introduced with the aid of *N,N′*-dicyclohexylcarbodiimide (DCC) or *N,N′*-diisopropylcarbodiimide (DIPCDI), plus 1-hydroxybenzotriazole (HOBt), in *N,N*-dimethylformamide (DMF) at 25 °C for 4 h, or in DMF–CH_2Cl_2 (1:1 v/v) overnight at 25 °C (compare to Table II, note *c*). For rigid functionalized supports such as kieselguhr-encapsulated polydimethylacrylamide (Pepsyn K), silica, or controlled pore glass, coupling of handle **1** was preferrably carried out by a recently reported method[20a] involving use of benzo-

(18) For a precedent to the reaction conditions used, see: Vowinkel, E.; Bartel, J. *Chem. Ber.* **1974**, *107*, 1221–1227.

(19) Carpino, L. A.; Mansour, E. M.; Cheng, C. H.; Williams, J. R.; MacDonald, R.; Knapczyk, J.; Carman, M.; Łopusiński, A. *J. Org. Chem.* **1983**, *48*, 661–665. This compound was also reported, without experimental details, in refs 10e and 10f.

triazolyl *N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)-1-hydroxybenzotriazole (HOBt)-*N*-methylmorpholine (NMM) in DMF (compare to Table III). By whatever coupling procedure was used, formation of the amide bond connecting the carboxyl of 1 to an amino support proceeded somewhat more slowly than peptide bond formation with the same reagents; nevertheless, attachment of 1 could be brought to completion readily, as judged by qualitative ninhydrin tests.[21] The Fmoc group was then removed from the PAL handle-resin, and stepwise elaboration[6] of peptide chains followed without difficulty using either Fmoc or Dts for temporary protection of N^{α}-amino groups, and applying any compatible coupling procedures (see Experimental Section for examples).

For small model peptides, final cleavage of *tert*-butyl side-chain protecting groups and of the anchoring linkage proceeded smoothly in trifluoroacetic acid–dichloromethane–dimethyl sulfide (14:5:1) (reagent A) at 25 °C for 2 h; cleavage yields were on the order of 80–95% and did not depend on the C-terminal residue.[2,9] Reagent A also readily removed the 2,4-6-trimethoxybenzyl (Tmob) protecting group from the C^{ω}-amides of asparagine and glutamine.[22] However, for complex peptides that contain several sensitive side-chain functionalities, or that include arginine residues blocked with the slowly cleaved 4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr) group[23] or somewhat more acid-labile 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) group,[24] alternative scavengers must be added to the cleavage cocktail. We found a freshly prepared mixture of trifluoroacetic acid–thioanisole–1,2-ethanedithiol–anisole (90:5:3:2) (reagent R), applied for 2–8 h at 25 °C, to be particularly convenient and general. Following essentially quantitative (>90%) release of chains with reagent R, the peptide products were normally precipitated with excess cold ethyl ether (10 volumes).

In the 5 years since the PAL handle was proposed,[2a] over a hundred C-terminal peptide amides of chain length 4–58 residues have been synthesized in these laboratories. Yields at all stages of the procedure were assessed in a number of cases by use of "internal reference" amino acids;[2b,7,25] in this way it was shown that the loading of the handle matched the initial substitution of amino sites on the support, and that all amino acid residues required in the target peptides were quantitatively incorporated. Purities of the products directly after cleavage were commensurate with the known scope and limitations of the overall synthesis protocols (i.e., deprotection/coupling procedures, side-chain protecting groups). All 20 naturally encoded amino acid residues have been incorporated. Furthermore, there were no differences in yields or purities of peptides derived from either of the pure isomeric handles 1 and 1′, or from the isomer mixture[2b] 1/1′.

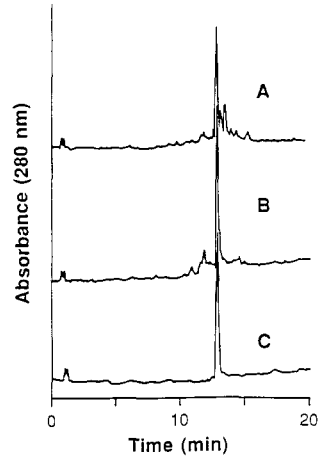


Figure 1. Analytical HPLC of samples from solid-phase syntheses of human gastrin-I. HPLC was performed with a Vydac C-18 reversed-phase column (4.6 × 250 mm); linear gradient over 20 min using CH_3CN and 0.01 N aqueous HCl from 1:9 to 7:3; flow rate 1.5 mL/min; UV absorbance 280 nm (identical profiles were observed at 215 nm). (A) Crude cleaved material, from a suboptimal synthesis starting with a Fmoc-PAL-MBHA-polystyrene support (details in last paragraph on gastrin, Experimental Section). (B) Crude cleaved material from synthesis starting with a Fmoc-PAL-Nle-MBHA-polystyrene support (full description in Experimental Section; the desired peptide corresponds to ≈85% of the total material). (C) Purified gastrin after a single MPLC run; see text and supplementary material for further characterization.

Solid-Phase Syntheses of Human Gastrin-I. The title molecule, sequence *p*Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2, provides a stringent test for any synthetic methodology because of the high density of acid-sensitive residues including the two tryptophans and the five consecutive glutamic acids.[13] Stepwise assembly was carried out several times both manually and on automated instrumentation. Fmoc was used for N^{α}-amino protection, and couplings were mediated by either DIPCDI plus HOBt in DMF–CH_2Cl_2 or by BOP–HOBt–NMM in DMF (Tables II and III). Amino acid ratios upon hydrolyses of the peptide-resins were in complete accord with expectation (supplementary material). Cleavage from the support (68% yield) was best achieved with reagent R to provide material in which the major component comigrated with authentic gastrin on HPLC (Figure 1, parts A and B). Purification by preparative medium-pressure reversed-phase liquid chromatography (MPLC) gave HPLC-pure gastrin-I (Figure 1C) in an overall isolated yield of 37%. The correct structure of purified gastrin-I was further supported by its amino acid composition and by the presence of quasi-molecular ions upon fast atom bombardment mass spectrometry (FABMS) (see the supplementary material).

Optimization of Cleavage Cocktails, and a Side Reaction at Tryptophan. Implicit in the description of the successful gastrin-I synthesis just reported, which featured cleavage with reagent R, are the substantially inferior results when a number of other cleavage cocktails (including reagent A) were tested on the *identical* protected peptide-resins. Complicating the issue, the exact mode of attachment of the PAL handle to the polymeric support made a difference in the chromatographic profile of crude gastrin-I (compare Figure 1, part A, with Figure 1, part B), even when reagent R was fixed for the cleavage.

(20) (a) The protocol used was reported by Hudson, D. *J. Org. Chem.* **1988**, *53*, 617–624. (b) BOP was introduced to peptide chemistry by Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219–1222. See also: (c) Le-Nguyen, D.; Heitz, A.; Castro, B. *J.Chem. Soc., Perkin Trans. 1* **1987**, 1915–1919. (d) Fournier, A.; Wang, C. T.; Felix, A. M. *Int. J. Peptide Protein Res.* **1988**, *31*, 86–97.

(21) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. *Anal. Biochem.* **1970**, *34*, 595–598.

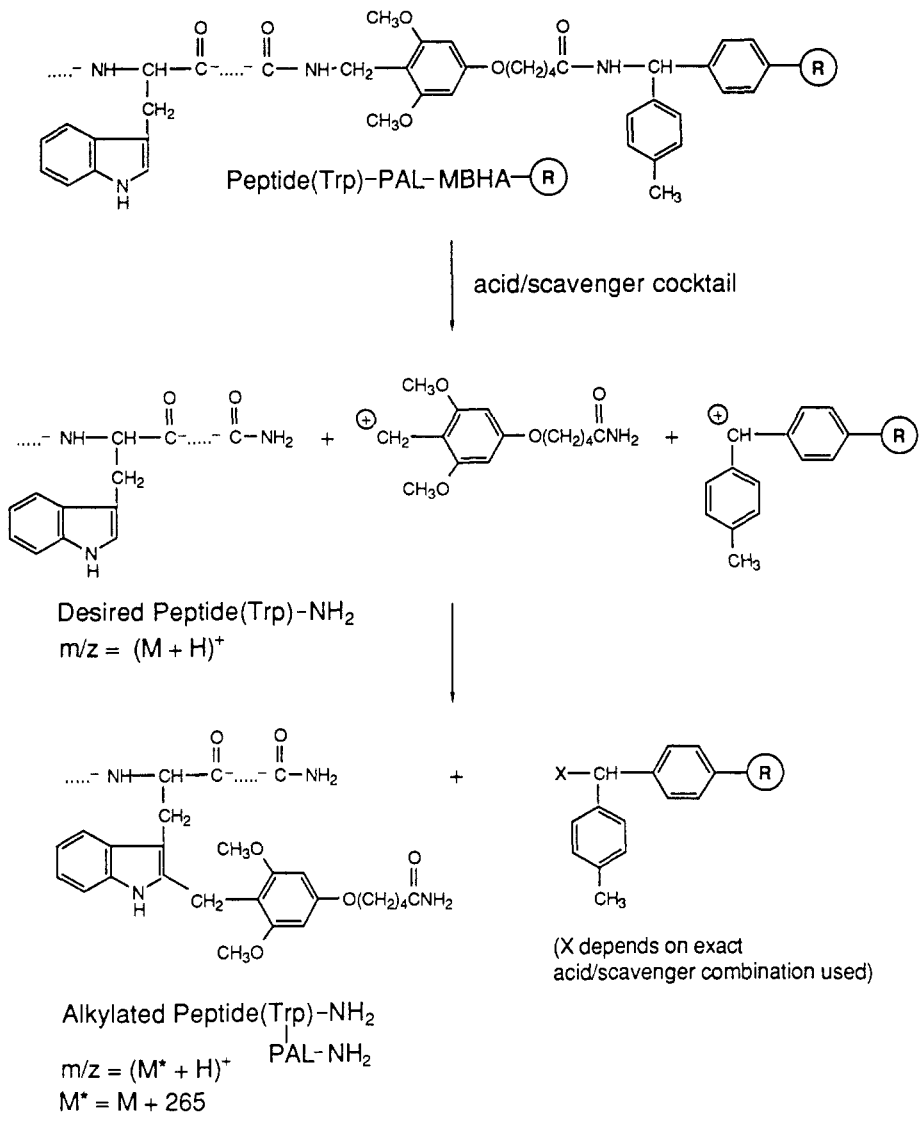
(22) The 2,4,6-trimethoxybenzyl (Tmob) protecting group for the ω-carboxamide side chains of asparagine and glutamine was reported by D. Hudson at the 10th American Peptide Symposium, St. Louis, May 23–28, 1987; see also Biosearch Technical Bulletin 9000-01 (1988). Appropriate N^{α}-Fmoc-protected derivatives are available from MilliGen/Biosearch and Bachem.

(23) (a) Fujino, F.; Wakimasu, M.; Kitada, C. *Chem. Pharm. Bull. Jpn.* **1981**, *29*, 2825–2831. (b) Atherton, E.; Sheppard, R. C.; Ward, P. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2065–2073. Cleavage of the Mtr group is unsuccessful with reagent A and requires 8 h at 25 °C with reagent R.

(24) Ramage, R.; Green, J. *Tetrahedron Lett.* **1987**, *28*, 2287–2290. The Pmc group is cleaved with reagent R in 3 h at 25 °C.

(25) (a) Atherton, E.; Clive, D. L.; Sheppard, R. C. *J. Am. Chem. Soc.* **1975**, *97*, 6584–6585. (b) Matsueda, G. R.; Haber, E. *Anal. Biochem.* **1980**, *104*, 215–227.

Scheme III



Concurrent with our work on the gastrin-I problem, we faced disappointing initial results on the synthesis of an adipokinetic hormone from *Tabanus atratus*.[26] The sequence *p*Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2 was assembled on a support where PAL handle 1 was attached *directly* to a *p*-methylbenzhydrylamine (MBHA) resin. Two major peaks were observed (Figure 2A), both of which were readily obtained in pure form by preparative HPLC. The earlier eluting peak proved to be the desired peptide, whereas the undesired byproduct was shown by high-field NMR and FABMS to *include* the PAL moiety (ending as an amide rather than as an acid) (details in supplementary material). The byproduct was not observed whatsoever as part of the crude cleavage product when the starting support was reformulated in either of two ways: (i) PAL handle 1 was attached to the aminoalkyl group of a Pepsyn K support (Figure 2B), or (ii) PAL handle 1 was attached onto the N^{α}-amino group of an "internal reference" amino acid (see the Experimental Section).

Formation of significant amounts of a PAL-containing byproduct was also observed in studies on the model peptide H-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala-NH_2

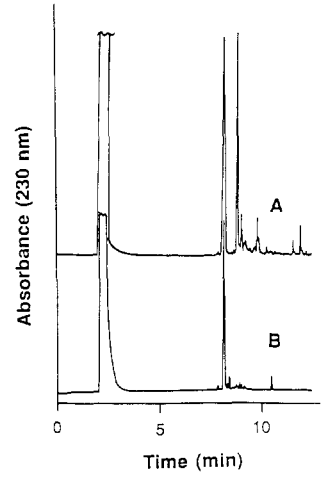


Figure 2. Analytical HPLC of *crude* samples from solid-phase syntheses of *Tabanus atratus*-adipokinetic hormone (ref 26). HPLC was performed on a Vydac C-18 reversed-phase column (4.6 × 250 mm); with a linear gradient starting at 3 min and continued over 17 min; using 0.05% CF_3COOH in CH_3CN and 0.05% aqueous CF_3COOH, from 1:19 to 20:0; flow rate 1.7 mL/min; UV absorbance 230 nm. The large peaks at the fronts of the chromatograms are solvent. Details on synthesis and cleavage are in Experimental Section. (A) Synthesis started with Fmoc-PAL-MBHA-polystyrene. (B) Synthesis started with Fmoc-PAL-Pepsyn K.

(26) Jaffe, H.; Raina, A. K.; Riley, C. T.; Fraser, B. A.; Nachman, R. J.; Vogel, V. W.; Zhang, Y. S.; Hayes, T. K. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 8161-8164.

Table I. Optimization of Scavengers for Acidolytic Cleavage of Tryptophan-Containing Peptide Amides from PAL Anchoring Linkage[a]

line	reagent/scavenger[b]	time, h	product distribution, % target peptide[c]	alkylated peptide[d]	others[e]
	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH$_2$				
1	CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	4	40	40	20
2	CF_3COOH–CH_2Cl_2–thioanisole–EDT–anisole (70:20:5:3:2)	2	48	37	15
3	CF_3COOH–CH_2Cl_2–thioanisole–EDT–anisole (70:20:5:3:2)	4	37	46	17
4	CF_3COOH–thiophenol (24:1)	4	21	66	13
5	CF_3COOH–CH_2Cl_2–indole (12:12:1)	4	7	22	71
6	CF_3COOH–CH_2Cl_2–benzyl mercaptan (14:5:1)	4	22	49	29
7	CF_3COOH–CH_2Cl_2–thioanisole–EDT–anisole (50:40:5:3:2)	2	42	38	20
	H-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala-NH$_2$				
8	CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	2	43	38	19
9	CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	8	34	45	21
10	CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	18	22	39	39
11	CF_3COOH–CH_2Cl_2 (7:3)	2	15	57	28
12	CF_3COOH–CH_2Cl_2–dimethyl sulfide (14:5:1) (reagent A)	2	42	44	14
13	CF_3COOH–CH_2Cl_2–EDT (28:10:1)	2	28	47	25
14	CF_3COOH–CH_2Cl_2–indole (28:10:1)	2	14	12	74
15	CF_3COOH–CH_2Cl_2–dimethyl phosphite (28:10:1)	2	25	46	29
16	CF_3COOH–water (24:1)	2	44	39	17
17	CF_3COOH–2-propanol (24:1)	2	25	49	9
18	CF_3COOH–ascorbic acid (39:1)	2	10	62	28
19	CF_3COOH–tri-*n*-butylphosphine (39:1)	2	13	48	39
20	CF_3COOH–phenol (19:1)	2	32	49	19

[a] The rationale of these studies is explained in text ref 27. Peptides were assembled by standard protocols with *tert*-butyl ethers and esters for side-chain protection. All cleavages were carried out at 25 °C, using the cleavage cocktail in column 2 for the time indicated in column 3. The PAL handle 1 was attached directly to an MBHA support, and cleavage yields were not determined in most cases. The crude product mixtures from the cleavages were subjected directly to HPLC analysis (e.g. Figure 2A), and the product distributions reported are uncorrected for relative absorbances of peptides. All of the raw HPLC data are in the supplementary material. [b] Ratios are given as v/v, except solids (e.g., indole, ascorbic acid) are given as w/v. All cleavage cocktails were prepared freshly immediately before use. [c] "Target peptide" refers to the sequence cited in italics before presentation of the cleavage data. For both tryptophan-containing peptides considered, the desired peptide was obtained pure by preparative HPLC, and showed satisfactory amino acid composition and FABMS spectra (see the supplementary material). [d] "Alkylated peptide" was obtained pure by preparative HPLC, and characterized by NMR (extra singlets characteristic of PAL at δ 3.77 and 6.21; change in aromatic region $\delta \approx 7.2$) and FABMS (ions at m/z 265 higher than corresponding ions in target peptide). The full characterization of the alkylated peptides is given in the supplementary material. These peaks were *entirely absent* when the target peptides were synthesized on reformulated supports in which the bond between PAL and the amino group on the support was *stable* to acid (see text, *Optimization of Cleavage Cocktails, and a Side Reaction at Tryptophan*). [e] "Other" refers to one or more remaining peaks (unidentified), normalized so that the sum of all peaks was 100%. In the experiment where H-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala-NH_2 was treated with reagent R as a function of time (first three lines for this peptide), one specific peak among "other" increased corresponding to the decrease in the desired peptide peak.

(again documented by NMR and FABMS; see the supplementary material). A likely explanation (Scheme III) for *all* results with tryptophan-containing peptides invokes two processes: (i) the stable carbonium ion generated by cleavage of the anchoring linkage alkylates tryptophan, probably at the 2-position of the indole side chain; (ii) the amide bond connecting the PAL valeryl group to the MBHA-resin cleaves also. Consistent with this idea is the absence of alkylated byproducts *released into solution* once a more acid-stable bond is used to attach the PAL carboxyl group to the support; in these latter cases a stable tryptophan-alkylated resin derivative results which is reflected by a lower overall cleavage yield but no significant reduction in the purity of the cleaved peptide.

The systems in which alkylation at tryptophan is favored by the sequence and where *furthermore* the alkylated species is released into solution (e.g., Figure 2A) are ideally suited[27] for a rigorous comparison (Table I) of the merits of a wide variety of scavengers, both new and from the literature.[2,28] Reagent R and related cocktails gave the highest levels of desired target peptide with respect to undesired byproducts. Nevertheless, the target peptides suffered some destruction upon extended exposure to reagent R. Previously advocated scavengers such as phenol, thiophenol, benzyl mercaptan, indole,[29] dimethyl phosphite, and tri-*n*-butylphosphine were surprisingly ineffective in suppressing tryptophan alkylation.[30]

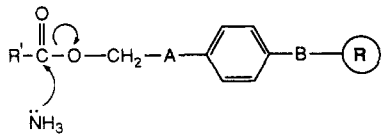
(27) The logic of these studies requires re-emphasis. On the one hand, with the proper formulation of the PAL-support, any putative tryptophan-alkylation byproduct must remain resin-bound. Such an approach solves unambiguously the practical problem of obtaining relatively homogeneous products by solid-phase peptide synthesis. However, to guage best the relative merits of various acid/scavenger combinations (Table I), we use those PAL-MBHA supports in which the tryptophan-alkylation byproducts are released into solution and can therefore be estimated by HPLC.

(28) Many leading references to the range of scavengers that have been proposed are found in ref 6b, Table IX, pp 72–75. The combination CF_3COOH–H_2O (19:1) has been favored by the Cambridge group, e.g., ref 32c (see later). The advantages of benzyl mercaptan were pointed out by Kemp, D. S.; Fotouhi, N.; Boyd, J. G.; Carey, R. I.; Ashton, C.; Hoare, J. *Int. J. Peptide Protein Res.* **1988**, *31*, 359–372.

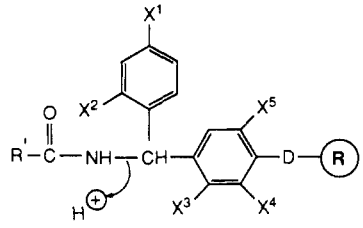
(29) The ineffectiveness of indole in the system reported in Table I stands in contrast to the usefulness of this additive in the human gastrin-I system. Thus, whereas cleavage of the protected gastrin-I-peptide-resin with CF_3COOH–CH_2Cl_2–dimethyl sulfide (14:5:1) (reagent A) gave an extremely heterogeneous crude product, results with CF_3COOH–CH_2Cl_2 (7:3) containing 2% (w/v) indole, 2 h, 25 °C, were very satisfactory and indistinguishable from the text results (Figure 1A) using reagent R.

(30) While this manuscript was undergoing review, we obtained preprints of two relevant studies which probed the tryptophan alkylation problem and the efficacy of various acid/scavenger combinations: (a) Gesellchen, P. D.; Rothenberger, R. B.; Dorman, D. E.; Paschal, J. W.; Elzey, T. K.; Campbell, C. S. In *Peptides; Chemistry, Structure and Biology Proceedings of the Eleventh American Peptide Symposium*; Rivier, J. E., Marshall, G. R., Eds.; Escom Science Publishers: Leiden, The Netherlands, 1990; pp 957–959. (b) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Peptide Protein Res.*, in press. Conclusions of these workers support our independent ones presented in this paper.

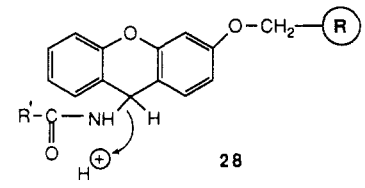
Scheme IV†



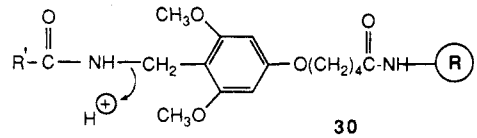
16 A, B = none (a)
17 A = (C=O), B = none (a)
18 A = none, B = (C=O)NH (b)



19 X^1, X^2, X^3, X^4, X^5 = H; D = none (a)
20 X^1 = CH_3; X^2, X^3, X^4, X^5 = H; D = none (a)
21 X^1, X^2 = CH_3O; X^3, X^4, X^5 = H; D = none (a)
22 X^1 = CH_3O; X^2, X^3 = H; X^4, X^5 = CH_3; D = $OCH_2(C=O)$ (b)
23 X^1, X^3 = CH_3O; X^2, X^4 = H; X^5 = $CH_2CH_2(C=O)$; D = H (c)
24 X^1, X^2 = CH_3O; X^3, X^4, X^5 = H; D = O (a)
25 X^1, X^2 = CH_3O; X^3, X^4, X^5 = H; D = $OCH_2(C=O)$ (b)
26 X^1, X^3 = CH_3O; X^2, X^4 = H; X^5 = $CH_2CH_2(C=O)$; D = OCH_3 (c)
27 X^1, X^2, X^3 = CH_3O, X^4, X^5 = H; D = $NH(C=O)CH_2CH_2(C=O)$ (b)
29 X^1 = NO_2; X^2, X^3, X^4, X^5 = H; D = none (a)



28



30

†Notes: Ⓡ is polymeric support, R'(C=O) is amino acyl or peptidyl. (a) Direct derivatization of the support supplies the functional group that will eventually give the peptide amide. (b) The functional group leading to the peptide amide is introduced as part of a handle. The linkage between the handle and support is generally a peptide bond between a carboxyl group on the handle and an amino group on the support, but occasionally it is an ester involving the carboxyl group of the handle. (c) This is a handle, but attachment is not through the para position as shown in the generic structure, but rather through the carboxyl of group X^5 in the meta position.

Comparative Studies of Acid-Sensitive Supports for C-Terminal Peptide Amides. Our preliminary reports on PAL[2] were followed in the literature[10] by proposals of other resin supports directed toward the same goal (structures in Scheme IV; see Discussion to this paper for complete appraisal). We recently obtained samples of two such supports that have become commercially available[11] and evaluated them in strictly controlled parallel syntheses (see text Figure 3 and supplementary Figure 2).

(31) Tatemoto, K.; Mutt, V. *Proc. Nat. Acad. Sci. U.S.A.* **1981**, *78*, 6603–6607.

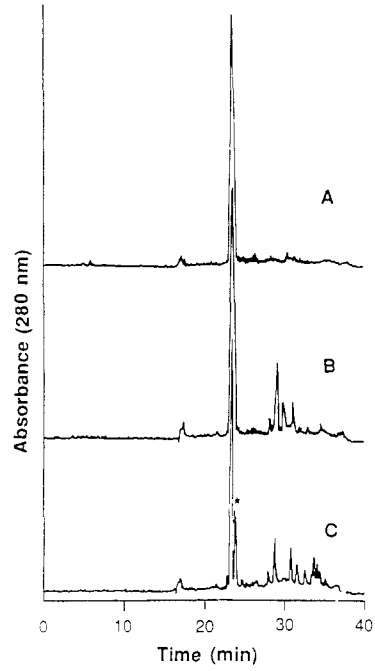


Figure 3. Analytical HPLC of *crude* intestinal peptide PHI porcine fragment (18–27) (see ref 31 for more information about this structure), prepared on a variety of supports. HPLC was performed on a Vydac C-18 reversed-phase column (4.6 × 250 mm); with a linear gradient over 30 min; using 0.036% CF_3COOH in CH_3CN and 0.045% aqueous CF_3COOH, from 1:19 to 13:1, flow rate 1.5 mL/min; UV absorbance 280 nm. (A) Starting with a 1% cross-linked MBHA-polystyrene (0.30 mmol/g), a Nle "internal reference" amino acid was added and then Fmoc-PAL (1) was introduced. All couplings were performed using the DIPCDI method (Table II) to assemble H-Ser(*t*Bu)-Ala-Lys-(Boc)-Lys(Boc)-Tyr(*t*Bu)-Leu-Glu(O-*t*Bu)-Ser(*t*Bu)-Leu-Ile-PAL-Nle-Ⓡ. Cleavage with reagent A (see text), 25 °C, 2 h, proceeded in 96% yield based on hydrolysis of the cleaved resin. During this cleavage, the peptide-resin took on a moderate yellow coloration. The amino acid composition of the crude peptide was: Ser, 1.78; Ala, 0.97; Lys, 2.02; Tyr, 1.03; Leu, 2.02; Glu, 1.02; Ile, 0.94. (B) Linker-AM (**25**) was attached to the identical support, and all further operations were carried out in parallel. Cleavage with reagent A was in 57% yield and produced a slightly orange resin. The amino acid composition of the crude peptide was: Ser, 1.82; Ala, 0.99; Lys, 1.98; Tyr, 0.97; Leu, 2.05; Glu, 1.03; Ile, 0.98. (C) Commercially available "Rink-resin" (structure **24**; see ref 10f) (0.55 mmol/g), lacking an "internal reference" amino acid (refs 2b, 7, 25), was applied in parallel with the other two experiments. Upon addition of reagent A, the resin immediately became burgundy red, and the overall cleavage yield was ≈70%. The peak on HPLC which is marked with an asterisk may be the corresponding peptide acid, but this point has not been proven conclusively. The amino acid composition of the crude peptide was: Ser, 1.80; Ala, 1.02; Lys, 2.05; Tyr, 0.97; Leu, 2.02; Glu, 0.98; Ile, 0.96.

Since the sequences chosen for these studies did not contain tryptophan, and since we wanted to emphasize any differences in the acid lability of the supports, all cleavages were performed with reagent A. Cleavage yields were appreciably better with PAL handle **1**, by comparison to Rink-resin (**24**) and Linker-AM (**25**). The relative homogeneities of the crude cleavage products were also somewhat better when starting with the PAL handle.

Discussion

Synthetic Strategies for C-Terminal Peptide Amides. Prior methods to prepare C-terminal peptide amides suffer from requiring either strong acid (e.g., anhydrous hydrogen fluoride) or strong base (e.g., ammonia) for final cleavage. Thus, peptides assembled by standard chemistry using *tert*-butyloxycarbonyl (Boc) for N^{α}-amino

protection and anchored to supports by benzyl esters[6] (Scheme IV, structure **16**) have been cleaved by ammonolysis,[32] a technique clearly limited to peptides lacking C^{ω}-benzyl-protected aspartate or glutamate. Ammonolysis has also been applied to cleave substituted benzyl ester or phenacyl ester anchors (Scheme IV, structures **17** and **18**) for peptides assembled by Fmoc or other chemistries; for these variations, C^{ω}-*tert*-butyl-protected aspartate or glutamate can be accommodated. Nevertheless, a number of serious side reactions observed with nucleophilic cleavages[6b,d] spurred the development[33] of supports with a benzhydrylamine theme (Scheme IV, structures **19–21**). These are coupled to N^{α}-protected amino acids via the free C^{α}-carboxyl group to provide benzhydrylamide linkages, which cleave in acid to furnish the desired carboxamides (note that the bond formed during the anchoring step is not identical, but rather adjacent, to the bond that is eventually cleaved). Such procedures are subject to the well-known but often manageable limitations[6b,d] associated with final acidolytic cleavage. For N^{α}-Boc/side-chain benzyl chemistry, the *p*-methylbenzhydrylamine (MBHA) resin of Matsueda and Stewart[33c] has served well. To go along with milder N^{α}-Fmoc/side-chain *tert*-butyl and related chemistries, several workers[10c,d,f-k] have recently investigated benzhydrylamine derivatives substituted with electron-donating alkoxy groups (Scheme IV, structures **21–27**), as well as resin-bound xanthenylamides[10e] (Scheme IV, structure **28**). Alternatively, Colombo[10b] has reported briefly on a *p*-nitrobenzyhydrylamine support (Scheme IV, structure **29**) which, simultaneous to removal of side-chain benzyl esters, is cleaved by catalytic transfer hydrogenolysis. Preparations of derivatives required for any of the aforementioned milder approaches have invariably involved a number of complexities, which include but are not limited to key steps failing to reach completion.

Our approach to C-terminal peptide amides has been to use crystalline PAL handle **1**, which is quantitatively coupled in a single step onto amino-functionalized supports to provide a general starting point of well-defined structure (Scheme IV, structure **30**) for peptide chain assembly. The Fmoc group protecting **1** is removed, and then, as with the benzhydrylamine and xanthydryl-based supports, the C-terminal residue is quantitatively coupled as its N^{α}-amino protected free C^{α}-carboxyl *acid*. Ultimately, acidolytic cleavage of the tris(alkoxy)benzylamide anchoring linkage **30** gives cleanly the desired amide. Cleavage yields with **30** are experimentally found to be better than those using alternative anchors (Scheme IV) which presumably have lower electron density in the methylene carbon adjacent to the amide nitrogen.

As is well accepted in other contexts,[6e,7,10l,34] the handle approach embodied here with compound **1** is greatly preferred over procedures in which the important chemical transformations are carried out on the resin (as is the case for structures **19**, **20**, **21**, and **24**; Scheme IV). This is because resin derivatization procedures inevitably result in the formation of resin-bound impurities which adversely impact on the efficacy of the support for synthetic applications.[35] Such problems are circumvented by the use of handles which have well-defined structure and purity. Additionally, handles can be adapted to a range of polymeric supports, whereas most of the published resin derivatization procedures are either incompatible with or destructive to polyamide-based supports.

We have previously discussed[6e,7] advantages to the use of preformed handles for the synthesis of C-terminal peptide *acids*. A counterpoint to those advantages stems from the need to purify a separate preformed derivative for each prospective C-terminal residue. In contrast, PAL handle **1** directed towards peptide amides is "universal" insofar as a *single* reagent suffices for all prospective C-terminal residues. The same assessment holds for Linker-AM (**25**), the handle variant of Rink-resin (**24**). Our comparative studies (Results; e.g., Figure 3) show that, of the commercially available approaches, use of PAL should provide peptide amide products with unsurpassed yields and purities.

Improved Preparations of the PAL Handle. Two efficient routes (Scheme II) have been worked out which overcome some of the shortcomings associated with an earlier procedure.[2b] Pure positional isomers can now be obtained by changing the order of steps, and the overall yield has been increased several-fold. Synthetic problems that were faced included the need to control the initial functionalization of the highly electrophilic aromatic ring, optimization of the alkylation step[17] that introduces an ω-carboxyl group for attachment to the support, and development of effective conditions for reductive conversion of an electron-rich carbonyl to an aminomethyl group. Similar difficulties have been observed with related recently proposed handles and resin derivatives (Scheme IV). Solubility considerations are also significant at several steps in the preparation of **1**.

In our earlier work,[2b] the required aminomethyl group was formed by applying to oxime acid **9** hydrogenolysis in ethanol, in the presence of platinum(IV) oxide and chloroform, according to Secrist and Logue.[36] Unfortunately, those conditions promoted esterification, so that the product was not the expected amino acid **10** but rather the amine ester **12** (obtained as its hydrochloride). Furthermore, several preliminary trials by catalytic transfer hydrogenation[37] failed to generate amino groups. We now find that hydrogenolysis of **9** using a palladium catalyst in the presence of acetic acid provides desired **10** (Scheme II, left side), thereby obviating the extra saponification step of the earlier route.[2b] Similarly, our revised reduction conditions smoothly converted oxime ester **11** to amine ester **12** (Scheme II, right side). The text approaches are particularly useful because attractive potential shortcuts to introduce an aminomethyl group[38,39] failed in the tris-

(32) (a) Bodanszky, M.; Sheehan, J. T. *Chem. Ind. (London)* **1964**, 1423–1424. (b) Manning, M.; Coy, E.; Sawyer, W. H. *Biochemistry* **1970**, *9*, 3925–3929. (c) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 538–546.

(33) (a) Pietta, P. G.; Marshall, G. R. *J. Chem. Soc. D* **1970**, 650–651. (b) Orlowski, R. C.; Walter, R.; Winkler, D. L. *J. Org. Chem.* **1976**, *41*, 3701–3705. (c) Matsueda, G. R.; Stewart, J. M. *Peptides* **1981**, *2*, 45–50. (d) Gaehde, S. A.; Matsueda, G. R. *Int. J. Pept. Protein Res.* **1981**, *18*, 451–458. (e) Tam, J. P. *J. Org. Chem.* **1985**, *50*, 5291–5298.

(34) (a) Mitchell, A. R.; Kent, S. B.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845–2852. (b) Tam, J. P.; Kent, S. B.; Wong, T. W.; Merrifield, R. B. *Synthesis* **1979**, 955–957 and references cited therein.

(35) An early example of this class of problems is given in ref 33b, where under suboptimal reaction conditions the desired benzhydrylamino-type supports were contaminated extensively by secondary amino functions (these become starting points for peptide chains that later cannot be cleaved). The chemistry reported in ref 10c appears to involve several incomplete steps; as re-examined in ref 10d, great care is required to minimize the levels of O-demethylation and loss of nitrogen from the resin-bound 2,4-dimethoxybenzhydrylamine.

(36) Secrist, J. A.; Logue, M. W. *J. Org. Chem.* **1972**, *37*, 355–356.

(37) (a) Kahn, S. A.; Sivanandaiah, K. M. *Synthesis* **1978**, 750–751. (b) ElAmin, B.; Anantharamaiah, G. M.; Royer, G. P.; Means, G. E. *J. Org. Chem.* **1979**, *44*, 3442–3444. (c) Anwer, M. K.; Spatola, A. F. *Synthesis* **1980**, 929–932.

(38) A variety of reductive amination conditions were explored, with the intent to convert aldehydes **7** or **8** directly to amines **12** or **10**. For precedents to reaction conditions, see: Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897–2904 and references cited therein.

(alkoxy)benzyl system.

Optimal Applications of the PAL Handle to Solid-Phase Peptide Synthesis. This paper provides experimental documentation of the successful use of PAL handle **1** in the preparation of a significant array of biologically important C-terminal peptide amides. For small and moderately sized peptides lacking arginine, cysteine, and tryptophan residues, advantageous results were obtained by final cleavage using a mixture of trifluoroacetic acid, dichloromethane, and dimethyl sulfide (reagent A). For more complicated peptide targets, we have demonstrated the critical influence of acid concentration and the nature of scavengers on the purities of materials obtained by cleavage of otherwise identical protected peptide-resins. Dimethyl sulfide (as it is found in reagent A), a reasonably effective scavenger for *tert*-butyl carbocations,[6b] slows down somewhat the rate of acidolysis of the PAL linkage, is not useful for removal of Mtr or Pmc from arginine, and does not significantly prevent alkylation at tryptophan. Indole, an obvious additive because of its resemblance to the side chain of tryptophan, leads to erratic results.[29] Our best results were obtained with a cleavage cocktail (reagent R) that includes anisole, thioanisole, and 1,2-ethanedithiol and was generally applicable to a range of challenging sequences.[30,40] Optimal side-chain protection was provided by *tert*-butyl-based ether, ester, and urethane derivatives for serine, threonine, tyrosine, aspartic and glutamic acids, and lysine, 2,4,6-trimethoxybenzyl (Tmob) for asparagine and glutamine,[22] Pmc for arginine,[24] and trityl for histidine[41] and cysteine; all of these groups were quantitatively removed without side reactions by reagent R. Furthermore, reagent R appears to suppress potential problems at unprotected methionine and tryptophan, and to minimize succinimide formation and $\alpha \rightarrow \beta$ rearrangement at aspartyl sequences.[42] We believe that these selections represent a comprehensive package for the solid-phase synthesis of any peptide as its C-terminal amide.

Conclusions

We have demonstrated a new general way to synthesize C-terminal peptide amides in high yields and good purities under mild conditions. The PAL handle **1**, best used in conjunction with an "internal reference" amino acid,[2b,7,25] is compatible with a wide array of polymeric supports, and with Fmoc/*tert*-butyl and related protection schemes. Furthermore, a single synthetic starting point suffices to accommodate *any* amino acid as the C-terminus. At the completion of the synthesis, optimized scavenger combinations are required to quench the very stable resin-bound carbonium ion which forms under the acidolytic cleavage conditions (per force, the scavengers also react with the soluble carbonium ions derived from the assortment of side-chain protecting groups). The described PAL-based methodology has proven itself in the past few years on numerous biologically significant peptides.

Experimental Section

Most of the materials and general synthetic and analytical procedures have been described in our earlier publications.[2b,3,7a-e,20a] ^{1}H NMR spectra were recorded at 300 MHz on either a Nicolet NT-300 WB or an IBM NR/300 instrument and at 200 MHz on an IBM NR/200 instrument using either $CDCl_3$ or CD_3SOCD_3 as solvents. ^{13}C NMR spectra were obtained at 75 or 50 MHz on the same instruments, and assignments of carbon resonances were facilitated by DEPT experiments. Fast atom bombardment mass spectrometry (FABMS) to characterize synthetic peptides was carried out on a VG 707E-HF instrument (details in the supplementary material), with glycerol or thioglycerol matrices being used to obtain both positive and negative ion spectra. Positive ion spectra often included not only $[M + H]^+$ ions, but also $[M + Na]^+$, $[M + K]^+$, and peaks with more than one metal (e.g., $[M + 2Na - H]^+$, $[M + Na + K - H]^+$). Sodiated or potassiated peaks were diminished after sample preparation using Waters C-18 "Sep-Pak"'s to remove salts and other impurities.[43] Elemental analyses were determined by M-H-W Laboratories, Phoenix, AZ.

Thin-layer chromatography was performed on Analtech or Merck silica gel GF plates (250 μm, 10 × 20 cm), and compounds were observed by fluorescence quenching and by spraying with a dilute ethanolic ninhydrin solution. Development was with: CA, chloroform–acetic acid (19:1); CMA, chloroform–methanol–acetic acid (18:1:1). Amino acid analyses and analytical HPLC were carried out on previously described Beckman, LKB, or Varian systems.

5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric Acid (1). Method A (Scheme II, left side). The amine precursor **10** (695 mg, 2.3 mmol) was suspended in a mixture of dioxane (9 mL) and 10% (w/v) aqueous Na_2CO_3 (9 mL), and Fmoc-azide (588 mg, 2.2 mmol) dissolved in dioxane (4.0 mL, + 0.5 mL for rinsing) was added with good stirring. The pH was maintained between 9.5 and 9.8 by addition of further 10% (w/v) aqueous Na_2CO_3, and the progress of the reaction was followed by TLC (CMA). The starting amine **10** was a streak at the origin, Fmoc-azide had R_f 0.85, and product **1** had R_f 0.72. The always heterogeneous reaction mixture was stirred for 3 h at 25 °C, poured into water (20 mL), extracted with ether (2 × 10 mL), and then carefully acidified to pH 3.0 by use of 6 N aqueous HCl (6.2 mL), under cooling. The aqueous solution was extracted with EtOAc (3 × 25 mL), and the combined organic phases were washed with saturated aqueous NaCl (3 × 15 mL), dried ($MgSO_4$), concentrated, and dried in vacuo over P_2O_5 to give an NMR- and TLC-pure white solid (730 mg, 65%).

A portion (560 mg) of this product was recrystallized by heating in methanol–chloroform (2:1 v/v; 40 mL) and then chilling to −20 °C. There was obtained the title product (530 mg, 94% recovery); mp 178–180 °C; ^{1}H NMR (CD_3SOCD_3) δ 7.89 (d, J = 7.3 Hz, 2 H), 7.72 (d, J = 7.3 Hz, 2 H), 7.41 (t, J = 7.3 Hz, 2 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.0 (broad t, 1 H, NH), 6.22 (s, 2 H, aromatic), 4.1–4.3 (m, 5 H), 4.00 (broad t, 2 H, OCH_2), 3.75 (s, 6 H, 2 × CH_3), 2.30 (broad t, 2 H), 1.6–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 174.7 (COOH), 160.1 (aryl C1), 159.2 (aryl C3 and C5), 156.0

(39) It was feasible to carry out Friedel–Crafts phthalimidomethylation of precursors **14** or **15** (structures in ref 17), although the reaction gave a complicated mixture of positional isomers plus the 2,4-diphthalimidomethyl species. Even so, the intended amine products **12** or **10** were apparently not formed upon attempted dephthaloylation by hydrazinolysis. For precedents to this chemistry, see: (a) Mitchell, A. R.; Kent, S. B. H.; Erickson, B. W.; Merrifield, R. B. *Tetrahedron Lett.* **1976**, 3795–3798. (b) Bryan, W. *J. Org. Chem.* **1986**, *51*, 3371–3372.

(40) Our working assumption (see Scheme III) is that a major problem in peptide sythesis with acid-sensitive supports is alkylation of tryptophan at the 2-position. See Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 1637–1640, for a report that an Mtr group originally protecting the δ-guanido function of arginine is transferred in acid to the 2-position of a neighboring tryptophan residue. This side reaction could be scavenged by 1,2-ethanedithiol, although upon extended treatment, the scavenger itself was incorporated as a dithioketal of a 2-(trifluoroacetyl)tryptophanyl residue. A further relevant precedent is due to Eberle, A. N.; Atherton, E.; Dryland, A.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1*, **1986**, 361–367. These workers observed low yields for acidolytic cleavage of a *p*-alkoxybenzyl ester anchoring linkage for a tryptophan-containing peptide, unless anisole and 1,2-ethanedithiol were added as scavengers; they rationalized their results in terms of back-addition of the detached peptide to a resin-bound carbonium ion.

(41) Sieber, P.; Riniker, B. *Tetrahedron Lett.* **1987**, *28*, 6031–6034.

(42) Based on clues from ref 23a, we designed the model peptide H-Arg-Trp-Met-Ser-Asp-Asn-Val-NH_2 which was assembled on a PAL-resin using *tert*-butyl and Mtr for protection of sensitive side chains. The peptide-resin was cleaved by a range of acid/scavenger combinations, and the ratio of desired peptide to succinimide peptide was determined by HPLC. Whereas with cocktails based on strong acids (e.g., trifluoromethanesulfonic acid), the succinimide represented over half of the total product, cleavage with reagent R gave only a single peak corresponding to the correct structure. Derek Hudson, unpublished observations, 1988.

(43) (a) Martin, S. A.; Costello, C. E.; Biemann, K. *Anal. Chem.* **1982**, *54*, 2362–2368. (b) Moon, D. C.; Kelley, J. A. *Biomed. Env. Mass Spec.* **1988**, *17*, 229–237.

(Fmoc-carbonyl), 144.2 (Fmoc-aryl C), 140.9 (Fmoc-aryl C), 127.7, 127.2, 125.5, and 120.2 (Fmoc-aryl CH), 106.2 (aryl C4), 91.4 (aryl C2 and C6), 67.4 (OCH_2), 65.6 (Fmoc-CH_2), 55.9 (CH_3O), 46.9 (fluorenyl C9), 33.7 (CH_2 α to COOH), 33.2 (CH_2N), 28.4 and 21.5 (valeryl side chain).

Anal. Calcd for $C_{29}H_{31}NO_7$, MW 505.57: C, 68.90; H, 6.18; N, 2.77. Found: C, 68.92; H, 6.16; N, 2.80.

Method B (Scheme II, right side). The oxime precursor **11** (0.65 g, 2.0 mmol) was suspended in 80% aqueous acetic acid (5 mL) in a Parr hydrogenation vessel, and 10% Pd/C (90 mg) was added. Hydrogenation proceeded at 50 psi (3 atm), 25 °C, 2 h, at which time complete reaction was ascertained by TLC (CA) (starting material R_f 0.56; product remains at origin and is ninhydrin positive). The solution was filtered and evaporated at 2 mm and 25 °C to provide an oil (0.68 g), which was suspended in a mixture of dioxane (7 mL) and 2 N aqueous NaOH (7 mL). After 1.5 h, TLC in EtOH indicated that saponification was complete, and the pH was adjusted with 6 N aqueous HCl to 9.5. Fmoc-azide (0.5 g, 2.0 mmol) dissolved in dioxane (4 mL) was added portionwise over 30 min. The mixture was allowed to stir for an additional 90 min, with the pH maintained at 9.5 by addition of 10% (w/v) aqueous Na_2CO_3. After a further 2.5 h, the mixture was diluted with water (20 mL), and EtOAc (25 mL) was added. Using 6 N aqueous HCl, the mixture was carefully acidified to pH 3.0 under cooling, and the organic phase was separated. The aqueous solution was further extracted with EtOAc (2 × 25 mL), and the combined organic phases were washed with saturated aqueous NaCl (2 × 20 mL), dried ($MgSO_4$), and evaporated to dryness to give an NMR-pure light yellow solid (1.0 g, 99%), which was recrystallized (two crops) first from dioxane–ethyl ether and then from ethanol: yield 0.85 g (85%); mp 168–172 °C; NMR same as by method A.

Anal. Calcd for $C_{29}H_{31}NO_7$, MW 505.57: C, 68.90; H, 6.18; N, 2.77. Found: C, 68.94; H, 6.15; N, 2.69.

For both methods A and B, comparable results were obtained by substituting Fmoc-succinimide for Fmoc-azide as the reagent to introduce protection onto the PAL aminomethyl group. Scale-up entailed some modifications in the workup (extractions, recrystallization); batches of pure material in excess of 100 g were obtained in 40–70% yields for the final step. Specifically for method B, some 9-fluorenylmethyl carbamate (**13**) was observed in the crude reaction product along with desired **1**, as discussed in the text.

5-(2-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric Acid (1′). The amine precursor **10′** (292 mg, 0.85 mmol) was converted, in analogous fashion to method A for **1**, to give a pale orange solid (282 mg, 66%), pure by NMR and TLC, R_f (CMA) 0.78. A portion (146 mg) of this product was recrystallized by dissolving in hot methanol (2 mL), filtering through glass wool, and allowing crystal formation at 25 °C. There was obtained the title product, a pinkish-white powder (103 mg, 71% recovery); mp 116–118 °C; ^{1}H NMR (CD_3SOCD_3) δ 7.90 (d, J = 7.2 Hz, 2 H), 7.72 (d, J = 7.2 Hz, 2 H), 7.42 (t, J = 7.2 Hz, 2 H), 7.31 (t, J = 7.2 Hz, 2 H), 7.0 (br, 1 H, NH), 6.23 (s, 2 H), 4.1–4.3 (m, 5 H), 3.97 (broad t, 2 H), 3.78 and 3.77 (two adjacent singlets of equal height, total 6 H), 2.25 (broad t, 2 H), 1.6–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 174.4 (COOH), 160.6, 159.1, and 158.5 (aryl C1, C3, and C5), 155.8 (Fmoc-carbonyl), 144.0 (Fmoc-aryl C), 140.7 (Fmoc-aryl C), 127.6, 127.0, 125.4 and 120.0 (Fmoc-aryl CH), 106.4 (aryl C2), 91.5 and 90.8 (aryl C4 and C6), 67.4 (OCH_2), 65.2 (Fmoc-CH_2), 55.4 (CH_3O), 46.5 (fluorenyl C9), 33.0 (CH_2 α to COOH and CH_2N), 27.9 and 20.8 (valeryl side chain).

Anal. Calcd for $C_{29}H_{31}NO_7$, MW 505.57: C, 68.90; H, 6.18; N, 2.77. Found: C, 68.76; H, 6.14; N, 2.78.

4-Formyl-3,5-dimethoxyphenol (3). The viscous mixture formed from 3,5-dimethoxyphenol (**2**) (20 g, 0.13 mol) and phosphorous oxychloride (24.2 mL, 0.26 mol) was mechanically stirred at 0 °C, and DMF (15 mL, 0.2 mol) was added portionwise over 0.5 h. The reaction mixture was stirred for an additional 15 h at 25 °C and then quenched by pouring over ice (300 g). The very acidic aqueous solution was washed with ethyl ether (3 × 200 mL), and the aqueous phase was filtered to remove a tan-colored residue (2.7 g, 11%) which by NMR was mainly 2-formyl-3,5-dimethoxyphenol (**4**); ^{1}H NMR (CD_3SOCD_3) δ 10.02, 6.16, 6.11, 3.87, 3.85; ^{13}C NMR (CD_3SOCD_3) δ 190.9 (formyl), 167.7, 164.7, and 163.2 (aryl C1, C3, C5, not further assigned), 104.9 (aryl C2), 92.7 (aryl C4), 90.2 (aryl C6), 55.6 (CH_3O), 55.5 (CH_3O), admixed with some 2,6-diformyl-3,5-dimethoxyphenol (**5**) (spectral data given below). The filtrate was diluted with further water (250 mL), and the pH was adjusted to 6.0 with 19 N aqueous NaOH (53 mL). A heavy precipitate formed, which was collected after 15 min on a Büchner funnel, washed with warm (32 °C) ethyl ether (4 × 100 mL) to extract away NMR-pure **4** (1.9 g after concentration, 8%), and dried in vacuo. Yield: 13.1 g (52%) of title product, a whitish tan powder (≈95% pure), which was dissolved in hot ethanol (250 mL) to provide after cooling an 85% recovery of NMR-pure (>99%) title product; mp 224–226 °C [lit.[14a] mp 222–224 °C]; ^{1}H NMR (CD_3SOCD_3) δ 10.16, 6.09, 3.76; ^{13}C NMR (CD_3SOCD_3) δ 184.8 (formyl), 164.8 (aryl C1), 163.2 (aryl C3 and C5), 106.4 (aryl C4), 91.4 (aryl C2 and C6), 55.2 (CH_3O).

Anal. Calcd for $C_9H_{10}O_4$, MW 182.18: C, 59.34; H, 5.53. Found: C, 59.17; H, 5.57.

Also, the aqueous filtrate at pH 6 after standing for an additional several days gave a pale beige powdery precipitate (0.2 g, 0.8%), which proved to be essentially pure 2,6-diformyl-3,5-dimethoxyphenol (**5**); ^{1}H NMR (CD_3SOCD_3) δ 10.13, 6.36, 4.02; EI mass spectrum (source 200 °C, solid probe 250 °C, 70 eV) m/z 210 ($M^{\bullet+}$, 90), 182 ($M^{\bullet+}$, – CO, 85), 181 ($M^{\bullet+}$ – COH, 64), 153 ($M^{\bullet+}$ – CO – COH, 100), 152 ($M^{\bullet+}$ – 2COH, 98). An analytical sample was obtained by recrystallization from hot methanol, mp 249–250 °C [lit.[16] mp 245–247 °C].

Anal. Calcd for $C_{10}H_{10}O_5$, MW 210.19: C, 57.14; H, 4.80. Found: C, 57.25; H, 4.82.

The title procedure for **3** was readily scaled up 10-fold, with similar yields and purities.

Ethyl 5-(4-Formyl-3,5-dimethoxyphenoxy)valerate (7). A suspension of 4-formyl-3,5-dimethoxyphenol (**3**) (3.64 g, 20 mmol) and potassium *tert*-butoxide (2.47 g, 22 mmol) in dry *N,N*-dimethylformamide (20 mL) was stirred briefly at 25 °C, and then ethyl 5-bromovalerate (**6**) (3.5 mL, 22 mmol) in dry DMF (20 mL) was added. The reaction mixture was heated at 110 °C for 5 h, following which solvent was removed at 60 °C and 2 mm. EtOAc (150 mL) was added, the inorganic salts were filtered off, and the organic extract was washed with water (30 mL), 2 N aqueous NaOH (20 mL), and saturated aqueous NaCl (2 × 20 mL), dried ($MgSO_4$), and evaporated to dryness to give an NMR- and TLC-pure yellow solid (5.7 g, 92%; comparable yields obtained on 0.5-mol scale). An analytical sample was crystallized from hot ether: mp 48–50 °C; R_f (CMA) 0.63; ^{1}H NMR ($CDCl_3$) δ 10.34 (s, 1 H), 6.06 (s, 2 H, ArH), 4.14 (q, J = 7.1 Hz, 2 H), 4.0–4.1 (m, 2 H), 3.87 (s, 6 H, 2 OCH_3), 2.39 (broad t, 2 H), 1.7–1.9 (m, 4 H), 1.26 (t, J = 7.1 Hz, 3 H); ^{1}H NMR (CD_3SOCD_3) δ 10.20 (s, 1 H), 6.26 (s, 2 H), 4.0–4.2 (m, 4 H), 3.82 (s, 6 H), 2.3–2.4 (m, 2 H), 1.6–1.8 (m, 4 H), 1.19 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (CD_3SOCD_3) δ 185.3 (formyl), 172.4 (ester COOH), 165.7 (aryl C1), 163.1 (aryl C3 and C5), 107.7 (aryl C4), 90.9 (aryl C2 and C6), 67.4 (OCH_2), 59.4 (CH_2 of ethyl), 55.7 (CH_3O), 32.8 (CH_2 α to COOH), 27.6 and 20.8 (valeryl side chain), 13.8 (CH_3 of ethyl); EI mass spectrum (source 200 °C, solid probe 200 °C, 20 eV) m/z 310 ($M^{\bullet+}$, 18), 265 (9), 181 (14), 129 [$EtO_2C(CH_2)_4^+$, 100], 101 (54), 83 (18).

Anal. Calcd for $C_{16}H_{22}O_6$, MW 310.35: C, 61.92; H, 7.15. Found: C, 62.06; H, 7.08.

The corresponding ortho isomer, **7′**, had ^{1}H NMR (CD_3SOCD_3) δ 10.22 (s, 1 H), 6.26 (s, 1 H), 6.25 (s, 1 H), 4.0–4.1 (m, 4 H), 3.86 (s, 3 H), 3.81 (s, 3 H), 1.7–1.9 (m, 4 H), 1.18 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (CD_3SOCD_3) δ 185.6 (formyl), 173.2 (ester COOH), 165.8 (aryl C1), 163.1 and 162.9 (aryl C3 and C5), 108.0 (aryl C2), 91.3 and 90.7 (aryl C4 and C6), 68.0 (OCH_2), 59.4 (CH_2 of ethyl), 55.8 and 55.6 (2 CH_3O), 32.8 (CH_2 α to COOH), 27.8 and 21.1 (valeryl side chain), 13.8 (CH_3 of ethyl).

5-(4-Formyl-3,5-dimethoxyphenoxy)valeric Acid (8). Method A. The pure ester precursor **7** (0.62 g, 2 mmol) was dissolved in methanol (4 mL), and 4 N aqueous NaOH (4 mL) was added. The solution was stirred for 1 h at 25 °C, diluted with water (4 mL), and concentrated at 25 °C (12 mm) to remove the alcohol. The aqueous phase was first extracted with EtOAc (2 × 10 mL) and then acidified with 12 N aqueous HCl (≈2.5 mL) to pH 3. The product was extracted into EtOAc (2 × 20 mL), and combined organic phases were washed with saturated aqueous NaCl (2 × 5 mL), dried ($MgSO_4$), and concentrated in vacuo to give a yellow powder (0.55 g, 96%), mp 129–132 °C, which was recrystallized from hot ethanol (86% recovery): small pale yellow

needles; mp 130–132 °C; R_f (CMA) 0.53; ^{1}H NMR ($CDCl_3$) δ 10.34 (s, 1 H), 6.06 (s, 2 H), 4.04 (broad t, 2 H), 3.87 (s, 6 H), 2.46 (broad t, 2 H), 1.8–1.9 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 185.8 (formyl), 174.5 (COOH), 165.6 (aryl C1), 163.6 (aryl C3 and C5), 108.0 (aryl C4), 91.3 (aryl C2 and C6), 67.9 (OCH_2), 56.2 (CH_3O), 33.4 (CH_2 α to COOH), 28.1 and 21.3 (valeryl side chain).

Anal. Calcd for $C_{14}H_{18}O_6$, MW 282.28: C, 59.56; H, 6.43. Found: C, 59.67; H, 6.41.

Method B. A mixture of 4-formyl-3,5-dimethoxyphenol (**3**) (3.65 g, 20 mmol) and potassium *tert*-butoxide (2.25 g, 20 mmol) in toluene (20 mL) was refluxed for 5 h under magnetic stirring. The toluene was removed by rotary evaporation, and ethyl 5-bromovalerate (**6**) (4.8 mL, 30 mmol) and DMF (50 mL) were added. The reaction mixture was stirred magnetically for 15 h at 110 °C, following which the solvent was removed at 60 °C (1 mm) to provide an oil (9.8 g) which lacked starting phenol but contained excess bromovalerate, as well as the ester precursor **7**. This entire oil was dissolved in 2 N aqueous NaOH–methanol (1:1, v/v) (130 mL). The solution was stirred for 30 min at 25 °C and then diluted with EtOAc (total 200 mL) and water (200 mL), and the organic phase was discarded. The aqueous phase was acidified with 12 N aqueous HCl to pH 1 and extracted with EtOAc (1 × 200 mL + 2 × 100 mL). The combined organic phases were washed with saturated aqueous NaCl (2 × 100 mL), dried ($MgSO_4$), and concentrated to give an orange powder (4.76 g, 85%). The product thus obtained was used directly for the next step without further purification, although an analytical sample was obtained by crystallization from hot acetone, hexane added at 25 °C for first crop, and further chilling to 4 °C for second crop. This gave a pale yellow solid (overall 80% recovery): mp 130–132 °C; ^{1}H NMR (CD_3SOCD_3) δ 10.20 (s, 1 H), 6.26 (s, 2 H), 4.1 (broad t, 2 H), 3.82 (s, 6 H), 2.3 (broad t, 2 H), 1.6–1.8 (m, 4 H).

Anal. Calcd for $C_{14}H_{18}O_6$, MW 282.29: C, 59.57; H, 6.43. Found: C, 59.62; H, 6.36.

Method C. A mixture of 4-formyl-3,5-dimethoxyphenol (**3**) (3.48 g, 19.0 mmol), K_2CO_3 (3.94 g, 28.5 mmol), and ethyl 5-bromovalerate (**6**) (5.96 g, 28.5 mmol) were refluxed (bp 95 °C) in 3-methyl-2-butanone (20 mL) for 21 h, filtered at 25 °C, and concentrated at 40 °C (2 mm). The resultant golden-brown oil (6.27 g), which included excess **6** but only trace **3**, was dissolved in methanol (32 mL), and 2 N aqueous NaOH (32 mL) was added. The solution was stirred for 30 min, diluted with H_2O (60 mL), partially concentrated at 30 °C (12 mm), and extracted with EtOAc (3 × 30 mL). The aqueous phase was brought to pH 2 with 12 N HCl (4.2 mL) and extracted with EtOAc (3 × 40 mL). The organic extracts were dried ($MgSO_4$) and concentrated to provide a semisolid (3.35 g, 69%); NMR as before.

5-(2-Formyl-3,5-dimethoxyphenoxy)valeric Acid (8′). A mixture of 2-formyl-3,5-dimethoxyphenol (**4**) (8.0 g, 44 mmol), K_2CO_3 (9.12 g, 66 mmol), and ethyl 5-bromovalerate (**6**) (13.8 g, 66 mmol) was reacted and worked up following method C above for **8**. The initial semisolid product (11.9 g, 96%) was dissolved in hot EtOAc (85 mL), and hexane (75 mL) was added portionwise to incipient turbidity. Crystals formed at 25 °C and were collected after 12 h: yield 6.7 g (55% overall for two steps); mp 103–104 °C; R_f (CA) 0.32; ^{1}H NMR (CD_3SOCD_3) δ 10.23 (s, 1 H), 6.26 (s, 1 H), 6.25 (s, 1 H), 4.05 (t, J = 5.9 Hz, 2 H), 3.86 (s, 3 H), 3.81 (s, 3 H), 2.29 (t, J = 7.1 Hz, 2 H), 1.6–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 185.7 (formyl), 174.3 (COOH), 165.9 (aryl C1), 163.1 (aryl C3 and C5), 108.1 (aryl C2), 91.3 and 90.8 (aryl C4 and C6), 68.1 (OCH_2), 55.9 and 55.7 (2 CH_3O), 33.2 (CH_2 α to COOH), 27.9 and 21.2 (valeryl side chain).

Anal. Calcd for $C_{14}H_{18}NO_6$, MW 282.28: C, 59.56; H, 6.43. Found: C, 59.71; H, 6.32.

5-(4-Hydroximinomethyl-3,5-dimethoxyphenoxy)valeric Acid (9). Hydroxylamine hydrochloride (0.6 g, 8.6 mmol) was dissolved in water (24 mL), and solid Na_2CO_3 (1.14 g) was added until a pH of 9.5 was obtained. This basic hydroxylamine solution was added to solid **8** (1.2 g, 4.3 mmol), and the suspension was stirred for 2 h with the pH maintained at 9.5 by addition of further Na_2CO_3. The resultant solution was acidified with 12 N aqueous HCl to pH 1 to provide title product as a pale yellow precipitate which was collected, washed with water (3 × 20 mL), and dried in vacuo over P_2O_5: yield 1.21 g (95%); mp 186–187 °C; R_f (CMA) 0.70; ^{1}H NMR (CD_3SOCD_3) δ 8.12 (s, 1 H), 6.26 (s, 2 H), 4.04 (t, 2 H), 3.77 (s, 6 H), 2.30 (t, 2 H), 1.7–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 174.1 (COOH), 161.3 (aryl Cl), 158.4 (aryl C3 and C5), 142.3 (aryl C4), 91.6 (aryl C2), 91.1 (aryl C6), 67.8 (OCH_2), 55.6 (CH_3O), 55.1 (CH_3O), 33.1 (CH_2 α to COOH), 27.9 and 20.9 (valeryl side chain).

Anal. Calcd for $C_{14}H_{19}NO_6$, MW 297.31: C, 56.56; H, 6.44; N, 4.71. Found: C, 56.63; H, 6.56; N, 4.79.

The title product **9** is very insoluble in acidic media, and precipitation of the pure product appears to be complete already at pH 5.5 (some buffering at this pH occurs during the acidification). The oxime **9** that remains in the mother liquor at pH 1–2 slowly hydrolyzes back to aldehyde **8**.

5-(2-Hydroximinomethyl-3,5-dimethoxyphenoxy)valeric Acid (9′). By the exactly analogous procedure to **9** above, **8′** (0.6 g, 2.1 mmol) was converted to the title product: yield 0.54 g (86%); mp 167–170 °C dec; R_f (CMA) 0.68; ^{1}H NMR (CD_3SOCD_3) δ 8.13 (s, 1 H), 6.26 (s, 2 H), 3.99 (t, 2 H), 3.80 (s, 3 H), 3.76 (s, 3 H), 2.28 (t, J = 7.1 Hz, 2 H), 1.6–1.8 (m, 4 H).

Anal. Calcd for $C_{14}H_{19}NO_6$, MW 297.31: C, 56.56; H, 6.44; N, 4.71. Found: C, 56.50; H, 6.53; N, 4.57.

5-(4-Aminomethyl-3,5-dimethoxyphenoxy)valeric Acid as the Acetate Salt (10). The oxime precursor **9** (0.8 g, 2.7 mmol) was suspended in 80% aqueous acetic acid (40 mL) in a Parr hydrogenation vessel, and 10% Pd/C (344 mg) was added. [These conditions represent a 3-fold increase over the concentration of a saturated solution of **9** in aqueous acetic acid; using the same amounts of material and the same volume of solvent, the oxime **9** was completely soluble in neat acetic acid and the overall outcome of the experiment was the same.] Hydrogenation proceeded at 50 psi (3 atm) and 25 °C for 2 h, at which time complete reaction was ascertained by TLC, R_f (CMA) 0.07 (origin). The solution was filtered and evaporated to dryness to give a dark gold oil (0.90 g, 96%), which solidified upon further standing; ^{1}H NMR (CD_3SOCD_3) δ 6.26 (s, 2 H), 4.0 (broad t, 2 H), 3.79 (s, 6 H) overlapping 3.7–3.9 (m, 2 H), 2.22 (broad t, 2 H), 1.85 (s, 3 H, acetate), 1.6–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 172.6 (COOH), 161.3 (aryl C1), 159.2 (aryl C3 and C5), 102.5 (aryl C4), 91.2 (aryl C2 and C6), 67.5 (OCH_2), 55.9 (CH_3O), 33.3 (CH_2 α to COOH), 31.4 (CH_2N), 28.3 and 22.0 (valeryl side chain).

Anal. Calcd for $C_{16}H_{25}NO_7$, MW 343.38: C, 55.97; H, 7.34; N, 4.08. Found: C, 55.88; H, 7.08; N, 3.97.

The corresponding ortho isomer **10′** was made in exactly analogous fashion, except the oxime precursor **9′** was *soluble* in 80% aqueous acetic acid at the solvent ratio cited.

Ethyl 5-(4-Hydroximinomethyl-3,5-dimethoxyphenoxy)-valerate (11). A suspension of aldehyde precursor **7** (4.65 g, 15 mmol) plus hydroxylamine hydrochloride (1.04 g, 16.5 mmol) in pyridine–H_2O (2:1 v/v; 11 mL) was stirred for 16 h at 25 °C. The reaction mixture was concentrated at 2 mm and 25 °C to give a light yellow crystalline mass, which was extracted for 15 min with 10% aqueous acetic acid (15 mL). The crystals were collected, washed with EtOAc (15 mL), and further washed with pentane (10 mL): yield 4.46 g (91%; same yield and purity on 0.5 mol scale; the starting material **7** need not be crystalline, but can be used directly from the preceding alkylation reaction of **3** with **6**); fluffy off-white powder; mp 158–160 °C; R_f (CA) 0.56 (versus 0.51 for starting material); ^{1}H NMR (CD_3SOCD_3) δ 8.12 (s, 1 H), 6.26 (s, 2 H), 4.06 (q, J = 7.1 Hz, 2 H) overlapping 4.0–4.1 (m, 2 H), 3.77 (s, 6 H), 2.38 (t, 2 H) 1.6–1.8 (m, 4 H), 1.19 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (CD_3SOCD_3) δ 172.8 (ester carbonyl), 160.9 (aryl C1), 159.2 (aryl C3 and C5), 142.4 (aldoxime), 102.0 (aryl C4), 91.5 (aryl C2 and C6), 67.3 (OCH_2), 59.7 (CH_2 of ethyl), 55.8 (CH_3O), 33.1 (CH_2 α to COOH), 28.0 and 21.2 (valeryl side chain), 14.2 (CH_3 of ethyl); EI mass spectrum (source 200 °C, solid probe 200 °C, 20 eV) m/z 325 ($M^{+\cdot}$, 8), 310 (6), 165 (7), 129 [$EtO_2C(CH_2)_4^+$, 100], 101 (51), 83 (16).

Anal. Calcd for $C_{16}H_{23}NO_6$, MW 325.35: C, 59.06; H, 7.12; N, 4.31. Found: C, 59.34; H, 7.26; N, 4.27.

Ethyl 5-(4-Aminomethyl-3,5-dimethoxyphenoxy)valerate (12). The oxime precursor **11** (0.33 g, 1.0 mmol) was suspended in 80% aqueous acetic acid (2.5 mL) and hydrogenated in the manner of the first few lines of method B for **1**. The title product was obtained as its acetate salt after evaporation: ^{1}H NMR (CD_3SOCD_3) δ 6.27 (s, 2 H), 4.06 (q, J = 7.1 Hz, 2 H), 4.0 (m, 2 H), 3.80 (s, 6 H) overlapping 3.7–3.9 (m, 2 H), 2.38 (broad t, 2 H), 1.88 (s, 3 H), 1.6–1.8 (m, 4 H), 1.19 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (CD_3SOCD_3) δ 172.9 (carbonyl), 161.4. (aryl C1), 159.3 (aryl

C3 and C5), 101.5 (aryl C4), 91.3 (aryl C2 and C6), 67.5 (OCH_2), 59.9 (CH_2 of ethyl), 56.0 (CH_3O), 33.3 (CH_2 α to COOH), 31.5 (CH_2N), 28.1 and 21.3 (valeryl side chain), 14.3 (CH_3 of ethyl). This material was taken up in a mixture of ether (25 mL), water (1 mL), and aqueous Na_2CO_3 (10% w/v; enough to liberate free amine). The organic phase was then washed with aqueous saturated NaCl (2 × 15 mL), dried ($MgSO_4$), and evaporated to dryness to give a white oil (0.30 g, 96%), which was further dried in vacuo over P_2O_5; ^1H NMR ($CDCl_3$) δ 6.10 (s, 2 H), 4.13 (q, J = 7.1 Hz, 2 H), 3.96 (m, 2 H), 3.80 (s, 6 H) overlapping 3.7–3.8 (m, 2 H), 2.38 (broad t, 2 H), 1.7–1.9 (m, 4 H), 1.25 (t, J = 7.1 Hz, 3 H).

Anal. Calcd for $C_{16}H_{25}NO_5$, MW 311.37: C, 61.71; H, 8.09; N, 4.50. Found: C, 61.90; H, 7.91; N, 4.22.

A portion of the amine was dissolved in ether and converted to the picrate, which was recrystallized from hot ethanol.

Anal. (for picrate) Calcd for $C_{22}H_{28}N_4O_{12}$, MW 540.48: C, 48.89; H, 5.22; N, 10.37. Found: C, 49.05; H, 5.16; N, 10.32.

The title reaction was carried out on a 0.2-mol scale, whereupon lengthier hydrogenation (i.e., overnight) was required. Progress was monitored by TLC in $CHCl_3$–acetic acid (4:1); R_f of starting **11**, 0.8; product **12**, 0.19 (yellow ninhydrin spot), ammonia, 0.06 (blue ninhydrin spot). As discussed elsewhere, the production of ammonia lowered the ultimate yield of PAL handle **1**. Ammonia could be removed by partitioning the product amine **12** between ether and water, with several further ether washes required to improve recovery of **12**.

9-Fluorenylmethyl Carbamate 13. This compound was observed in several instances as a byproduct arising along the way from **11** (via **12**) to **1**. It remains in the mother liquor during crystallization of **1** from EtOAc, although it is very insoluble in (and crystallizes from) ethanol; white solid; mp 192–195 °C (lit.[19] mp 200–201 °C); ^1H NMR (CD_3SOCD_3) δ 7.90 (d, J = 6.8 Hz, 2 H), 7.70 (d, J = 7.0 Hz, 2 H), 7.43 (t, J = 7.2, 2 H), 7.34 (t, J = 7.3, 2 H), 6.6 (br, 2 H, NH_2), 4.25 (apparent s, 3 H); ^{13}C NMR (CD_3SOCD_3) δ 156.6 (Fmoc-carbonyl), 144.0 and 140.8 (fluorenyl aryl C), 127.6, 127.1, 125.2, and 120.1 (fluorenyl aryl CH), 65.0 (Fmoc-CH_2), 46.8 (fluorenyl C9); EI mass spectrum (source 200 °C, solid probe 175 °C, 20 eV) m/z 239 ($M^{\bullet+}$, 2), 196 ($M^{\bullet+}$ – HNCO, 15), 178 ($M^{\bullet+}$ – CO_2 – NH_3, 100), 166 (37), 165 (43).

Anal. Calcd for $C_{15}H_{13}NO_2$, MW 239.27: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.25; H, 5.58; N, 5.63.

Ethyl 5-(3,5-Dimethoxyphenoxy)valerate (14). A mixture of 3,5-dimethoxyphenol (**2**) (3.0 g, 19.5 mmol), potassium tert-butoxide (2.4 g, 21.4 mmol), and ethyl 5-bromovalerate (**6**) (3.4 mL, 21.4 mmol) in dry *N,N*-dimethylformamide (12 mL) was heated for 4.5 h at 110 °C, at which point TLC in benzene–ethanol (4:1) indicated completion of the reaction (R_f 0.73 for product, 0.66 for starting phenol). Solvent was removed at 60 °C (2 mm), EtOAc (50 mL) was added, and the inorganic salts were filtered off. The organic extract was washed with 20% (w/v) aqueous NaCl (2 × 10 mL), 2 N aqueous NaOH (2 × 15 mL) and 20% (w/v) aqueous NaCl (2 × 15 mL), dried ($MgSO_4$), and evaporated to dryness to provide a gold oil which solidified completely after several days when maintained under high vacuum, and then brought to 4 °C: yield (3.8 g, 69%); mp 29–30 °C; R_f ($CHCl_3$) 0.4; ^1H NMR (CD_3SOCD_3) δ 6.08 (s, 3 H), 4.06 (q, J = 7.1 Hz, 2 H), 3.92 (broad t, J = 6.4 Hz, 2 H), 3.71 (s, 6 H), 2.36 (broad t, J = 6.7 Hz, 2 H), 1.6–1.8 (m, 4 H), 1.19 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (DMSO) δ 172.9 (ester carbonyl), 161.3. (aryl C1), 160.7 (aryl C3 and C5), 93.4 (aryl C2 and C6), 93.0 (aryl C4), 67.2 (OCH_2), 59.8 (CH_2 of ethyl), 55.2 (CH_3O), 33.3 (CH_2 α to COOH), 28.2 and 21.4 (valeryl side chain), 14.2 (CH_3 of ethyl).

Anal. Calcd for $C_{15}H_{22}O_5$, MW 282.33: C, 63.81; H, 7.86. Found: C, 64.81; H, 7.85.

5-(3,5-Dimethoxyphenoxy)valeric Acid (15). The same saponification procedure already reported for the conversion of **7** to **8** was applied to compound **14**. This gave the solid title product: mp 99–102 °C; ^1H NMR (CD_3SOCD_3) δ 6.08 (s, 3 H), 3.92 (broad t, J = 6.1 Hz, 2 H), 3.70 (s, 6 H), 2.28 (broad t, J = 6.8 Hz, 2 H), 1.5–1.8 (m, 4 H); ^{13}C NMR (CD_3SOCD_3) δ 174.5 (COOH), 161.3 (aryl C3 and C5), 160.7 (aryl C1), 93.4 (aryl C2 and C6), 93.0 (aryl C4), 67.3 (OCH_2), 55.2 (CH_3O) 33.4 (CH_2 α to COOH), 28.3 and 21.4 (valeryl side chain).

Anal. Calcd for $C_{13}H_{18}O_5$, MW 254.27: C, 61.40; H, 7.13. Found: C, 61.53; H, 7.22.

Table II. Deprotection/Coupling Cycle Using *N,N'*-Diisopropylcarbodiimide[a]

operation number	reagent, solvent	time, min
1	CH_2Cl_2 washes	5 × 0.3
2	piperidine–DMF (3:7, v/v)	1 × 2 1 × 8
3	CH_2Cl_2 washes	5 × 0.3
4	AA/DIPCDI (1:1) in CH_2Cl_2–DMF (1:1, v/v)[b,c]	60[d]
5	DMF washes	3 × 0.3

[a] Protocol for manual operation. Abbreviations are listed in ref 1. [b] The N^{α}-Fmoc-protected amino acids and DIPCDI were equimolar (net ≈0.1 M each), and in 3–7-fold excess over growing chains on the peptide resin (0.25 mmol/g). For the introduction of asparagine or glutamine, HOBt (1 equiv) was dissolved in DMF, and this solution was used to dissolve Fmoc-Asn-OH or Fmoc-Gln-OH respectively. This DMF solution was added to the deprotected peptide-resin, followed by a solution of DIPCDI (1 equiv) in CH_2Cl_2 (equal volume), so that the net CH_2Cl_2–DMF solvent was 1:1 (v/v). [c] In some instances, a DIPCDI–HOBt protocol was followed instead of title DIPCDI protocol. Thus, the technique of footnote b was followed for all N^{α}-Fmoc-protected amino acids. [d] Qualitative ninhydrin tests (ref 21) performed at this point verified that the coupling reaction had gone to completion.

PAL-Resins for Solid-Phase Peptide Synthesis. As outlined in the Results, a wide range of procedures were successful. To provide a single example, Fmoc-PAL derivative **1** (297 mg, 0.59 mmol) and HOBt (80 mg, 0.59 mmol) were dissolved in DMF (10 mL) and added to a norleucyl ("internal reference") amidomethyl-poly(styrene-co-1% divinylbenzene) resin (0.75 g, 0.29 mmol). This was followed by *N,N'*-diisopropylcarbodiimide (DIPCDI) (93 μL, 0.59 mmol), and the mixture was shaken at 25 °C to effect coupling. Ninhydrin tests[21] on resin aliquots were conducted after 1 h (clearly positive), 2 h (positive), 3 h (slightly positive), and 4 h (almost negative). The resin was then filtered and washed with CH_2Cl_2, and finally HOAc (60 μL, 1 mmol) and DIPCDI (157 μL, 1 mmol) were added and shaken for 15 min (in many cases, such an acetylation is not necessary). The resin was then filtered, washed with DMF, CH_2Cl_2, and CH_3OH (3 × 6 mL × 1 min per solvent), and dried at 2 mm over P_2O_5. To conserve handle **1**, lower excesses (e.g., 1.2–1.5 equiv) compensated by longer reaction times (e.g., overnight at 25 °C), were also satisfactory.

Stepwise solid-phase peptide synthesis then proceeded as described herein (Tables II, III, and related protocols). The Fmoc group is completely and preferentially removed from the handle with piperidine–DMF (1:1, v/v), 1 min prewash + 10 min treatment (or related conditions as in Tables II and III). *We can no longer recommend* our earlier reported[2b] conditions of manual Fmoc removal, i.e. piperidine–CH_2Cl_2 (1:1, v/v), 3 × 2 min (the reagent develops a precipitate of amine salts after short standing, and is therefore particularly inappropriate for automated instruments). A *single* case was observed when with the *inferior* piperidine–CH_2Cl_2 (1:1) reagent, only about 60% of the Fmoc group was removed (this would have the undesirable consequence that later in the synthesis, chains lacking residues from the C-terminal may become initiated). This result shows that the handle Fmoc group is somewhat harder to remove completely than a normal amino acid bound Fmoc group, although again it must be emphasized that with piperidine–DMF (1:1), Fmoc removal is complete. In fact, the "internal reference" amino acid technique[2b,7,25] showed that with optimal protocols, nearly quantitative yields were obtained for all steps, including introduction of the handle, removal of the handle Fmoc group, and stepwise incorporation of Fmoc-protected amino acid derivatives.

Acyl Carrier Protein (65–74) Amide (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2).[44] Fmoc-PAL (**1**) was linked to kieselguhr-encapsulated polydimethylacrylamide (Pepsyn K)

(44) The acyl carrier protein (65–74) decapeptide is considered a "difficult" sequence for stepwise chain assembly and was first described by: Hancock, W. S.; Prescott, D. J.; Vagelos, P. R.; Marshall, G. R. *J. Org. Chem.* **1973**, *38*, 774–781. The retro-sequence is especially challenging, as noted by one of the authors (D.H.), unpublished observations, 1988.

Table III. Automated Deprotection/Coupling Cycle Using Benzotriazolyl *N*-Oxytris(dimethylamino)phosphonium Hexafluorophosphate–1-Hydroxybenzotriazole–*N*-Methylmorpholine[a]

operation number	reagent, solvent	time, min
1	CH_2Cl_2–DMF (1:1, v/v) washes	10 × 0.3
2	piperidine–DMF–toluene (6:7:7, v/v/v)	1 × 2 1 × 8
3	CH_2Cl_2–DMF (1:1, v/v) washes	10 × 0.3
4	AA/BOP/HOBt/NMM (1:1:1:1) in DMF[b,c]	60 or 120[d]

[a] These operations were performed automatically on a MilliGen/Biosearch Model 9600 Peptide Synthesizer. Protocol developed by D. Hudson (ref 20a). Abbreviations are listed in ref 1. [b] The indicated equimolar reagents were used in 8-fold excess over growing chains on the peptide-resin (0.25 mmol/g). The N^{α}-Fmoc-protected amino acid, BOP reagent, and HOBt were stored as solids in the reservoirs of the instrument; they were dissolved and activated by addition of equimolar NMM (0.2 M) in DMF. After 2 min at 25 °C, this homogeneous "preactivated" mixture was added directly to the deprotected peptide-resin. [c] An alternative automated procedure (compare to Table II, note *c*) used solutions of N^{α}-Fmoc-protected amino acids (0.4 M) in DMF containing equimolar HOBt, which were mixed with an equal volume of DIPCDI (0.4 M) in CH_2Cl_2. After 10 min at 25 °C, this "preactivated" solution was transferred to the reaction vessel containing the deprotected peptide-resin. [d] Coupling times were determined by a computerized "expert" system which analyzed the sequence with respect to steric, conformational, and size parameters.

(0.20 mmol/g), and all couplings of Fmoc-protected amino acid derivatives were performed automatically using the BOP–HOBt–NMM method [Table III; except Fmoc removal was with piperidine–DMF (3:7, v/v)]. The β- and γ-carboxamide functions of asparagine and glutamine, respectively, were protected as their Tmob derivatives,[22] and the *tert*-butyl ether and ester groups respectively protected the side chains of tyrosine and aspartic acid. Cleavage of the completed peptide-resin was achieved with freshly prepared reagent R, CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (1 mL used per 100 mg peptide-resin), 25 °C, 2 h, following which resin was removed by filtration through a disposable pipette with a glass wool plug, and the cleaved resin was washed with further reagent R (2 × 0.5 mL per 100 mg of resin). The peptide was precipitated from the combined filtrate and washings by addition of 10 volumes of cold anhydrous ethyl ether. The amino acid composition of the crude cleaved peptide was: Val, 1.02; Glx, 1.05; Ala, 2.00; Ile, 2.18; Tyr, 1.06; Asx, 2.12; Gly, 1.04. The crude peptide was solubilized by sonication in DMF–H_2O (1:1, v/v) and revealed a single major peak (>90%) by analytical HPLC (supplementary Figure 1).

Results were essentially the same with a 1% cross-linked polystyrene support, and with an automated coupling protocol based on DIPCDI in CH_2Cl_2–DMF (1:1, v/v) (Table II), regardless of the absence or presence of HOBt as an additive (Table II, notes *b*, *c*; Table III, note *c*). Syntheses were also carried out on a variety of rigid support materials including silicas, ceramics, and Corning controlled pore glasses. The best results from this set were obtained with Waters Porasil Type B silica (100–200-Å pores, 100 μmol/g aminopropyl groups), although this support was unsuitable for peptides containing in excess of 15 residues, presumably due to steric reasons.

Retro-Acyl Carrier Protein (74–65) (H-Gly-Asn-Ile-Tyr-Asp-Ile-Ala-Ala-Gln-Val-NH_2).[44] Starting with a 1% cross-linked MBHA-polystyrene (0.30 mmol/g), a Nle "internal reference" amino acid was added and then Fmoc-PAL (1) was introduced. For chain assembly by the DIPCDI method (Table II), the β- and γ-carboxamide functions of asparagine and glutamine were left unprotected, and the side chains of tyrosine and aspartic acid were protected as before. Cleavage with reagent A, CF_3COOH–CH_2Cl_2–dimethyl sulfide (14:5:1), 25 °C, 2 h, proceeded in 95% yield based on hydrolysis of the cleaved resin. The amino acid composition of the crude cleaved peptide was: Gly, 0.99; Asx, 1.90; Ile, 2.03; Tyr, 1.08; Ala, 2.00; Glx, 1.00; Val, 0.97. Alternatively, Linker-AM (**25**) was attached to the identical Nle-MBHA support, and all further operations were carried out in parallel. Cleavage with reagent A was in 30% yield to give material with amino acid composition: Gly, 0.97; Asx, 1.96; Ile, 2.07; Tyr, 1.03; Ala, 2.02; Glx, 0.98; Val, 0.97. Analytical HPLC of the crude peptide made with either handle revealed in each case a major peak (>90%), and comparable levels of low-level byproducts (see supplementary Figure 2).

Luteinizing Hormone-Releasing Hormone (LH-RH) (*p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Synthetic details were essentially as already given in the first paragraph of the acyl carrier protein (65–74) amide description. Side chains of arginine, tyrosine, and histidine were protected respectively with Pmc,[24] *tert*-butyl, and Trt,[41] and coupling followed Table III. The crude cleaved peptide had amino acid composition: Glu, 1.00; His, 1.01; Ser, 0.98; Tyr, 0.96; Gly, 1.89; Leu, 0.96; Arg, 0.98; Pro, 1.04. Analytical HPLC showed a major peak (≈80% purity). Tryptophan alkylation (compare to Scheme III, Figure 2), although possible in principle, does not appear to be significant in *this* sequence.

Human Gastrin-I (*p*Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2).[12,13] Chain assembly was carried out on a MilliGen/Biosearch Model 9600 Peptide Synthesizer, starting with an Fmoc-PAL-Nle-MBHA-polystyrene-resin (0.5 g, 0.25 mmol/g) and using N^{α}-Fmoc-amino acids (1.0 mmol each, 8.0 equiv) with a BOP–HOBt–NMM protocol (Table III). The β- and γ-carboxyl functions of aspartic and glutamic acids respectively were protected as their *tert*-butyl esters, and the phenolic side chain of tyrosine was protected as the *O*-*tert*-butyl ether. Upon hydrolysis in 12 N HCl–propionic acid (1:1 v/v) + 2 drops liquified phenol, 130 °C, 3 h, the completed peptide-resin included all of the amino acids in the expected ratios: Glu, 6.05; Gly, 1.93; Pro, 1.00; Leu; 0.92; Ala, 1.01; Tyr, 1.05; Met, 1.01; Asp, 0.97; Phe, 0.97; Nle, 0.95.

A portion of this peptide-resin (50 mg, 5.9 μmol) was treated with freshly prepared reagent R: CF_3COOH–thioanisole–EDT–anisole (90:5:3:2) (3 mL) in a solid-phase reaction vessel for 3 h at 25 °C. The filtrate was expressed from the vessel with positive nitrogen pressure, and the cleaved resin was washed with further reagent R (3 mL). Ice-cold ethyl ether (≈9 mL) was then added to the combined filtrates (≈6 mL) to induce cloudiness. The mixture was maintained at 5 °C for 10 min and then centrifuged to collect a fluffy white precipitate containing most of the peptide product (≈10% of the peptide material remained in the supernatant). The precipitate was washed with ether (3 × 5 mL), dissolved in glacial acetic acid (8 mL), and lyophilized to give a white solid (≈7 mg; cleavage yield 68% based on amino acid analyses both on the isolated product and on the cleaved resin). Purity at this stage was ≈85% (Figure 1B). A portion (5.3 mg, 2.54 μmol) of this material was dissolved in 2.0 mL of 0.05 M ammonium acetate buffer, pH 6.8, and applied to a Merck Lobar ARP-8 column. Elution was with a convex gradient formed from 500 mL each of 1:9 and 1:3 mixtures of CH_3CN and 0.05 M ammonium acetate buffer, pH 6.8; flow rate 300 mL/h; UV detection at 214 nm. The major peak corresponded to pure gastrin-I (1.40 μmol, 55% yield for the chromatography) and was characterized by analytical HPLC (Figure 1C), and FABMS (summarized in the following paragraph, see the supplementary material for actual spectra and further details). The purified gastrin-I had the following amino acid composition: Glu, 5.89; Gly, 2.18; Pro, 0.85; Leu; 1.06; Ala, 1.07; Tyr, 1.03; Met, 1.04; Asp, 1.09; Phe, 0.96.

Human gastrin-I, $C_{97}H_{124}N_{20}O_{31}S$, has a nominal mass of 2096 amu and a calculated exact monoisotopic mass of 2096.8432. Furthermore, the mass of the molecular ion containing 96 ^{12}C and 1 ^{13}C is 2097.8466, predicted to be in slightly greater abundance than the monoisotopic ion. The positive ion FABMS spectrum showed $[M + H]^+$ ions, agreeing with the calculated intensities at m/z 2097.9, 2098.9, 2099.9, and 2100.9. Smaller $[M + Na]^+$ peaks were observed at m/z 2119.8, 2121.0, and 2121.8. The negative ion FABMS spectrum gave $[M - H]^-$ at m/z 2095.9, together with isotopes. We also obtained several samples which had accumulated substantial levels of metal, to the point that the $[M + H]^+$ ions were insignificant and replaced by $[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H)^+$, $[M + Na + K - H]^+$, etc. Such samples were dissolved in 0.5 mL of 0.05 M ammonium acetate buffer, pH 6.8, and applied to a *new* C-18 Sep-Pak which had been

prewashed with HPLC-grade CH_3CN (3 mL) and 0.01 N aqueous HCl (3 mL). Salt impurities were removed by washing with 0.01 N aqueous HCl (2 × 3 mL), and the peptide was eluted with CH_3CN–0.01 N aqueous HCl (1:1 v/v) (2 × 3 mL). The filtrate was checked by analytical HPLC and lyophilized. In order to prevent recontamination with salts, all glassware for this procedure was washed with chromic acid, varying grades of water culminating with NANOpure water (Sybron/Barnstead water purification system), and HPLC-grade methanol.

An earlier synthesis, carried out the same way but starting with an Fmoc-PAL-MBHA-resin, gave similar results (cleavage yield 46%). In this case, reversed-phase MPLC gave not only the major desired peak corresponding to human gastrin-I, but also a later eluting byproduct which represented ≈25% of the UV absorbance (Figure 1A). The byproduct was isolated by MPLC (supplementary Figure 4) and thereby obtained as a single peak in HPLC; it contained all of the amino acids of desired gastrin in the approximately correct ratio: Glu, 5.26; Gly, 2.51; Pro, 0.76; Leu; 1.11; Ala, 1.07; Tyr, 0.99; Met, 1.31; Asp, 1.28; Phe, 0.71. The small amount of material obtained prevented definitive conclusions, but preliminary mass spectrometric studies suggest that this byproduct involves alkylation at one of the tryptophan residues, along the lines documented carefully for other peptides (see Scheme III and accompanying text).

***Tabanus atratus* Adipokinetic Hormone (*p*Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2).**[26] Several syntheses and cleavages were carried out to delineate the tryptophan alkylation problem (Figure 2, Scheme III, Table I, ref 27). Initially, Fmoc-PAL (1) was linked to MBHA-polystyrene (0.30 mmol/g), and further procedures were as already stated in the first paragraph of the acyl carrier protein (65–74) amide description. Analytical HPLC (Figure 2A) showed two major peaks, desired and tryptophan-alkylated (further characterization in the supplementary material). However, when Fmoc-PAL (1) was linked directly to kieselguhr-encapsulated polydimethylacrylamide (0.20 mmol/g) (Pepsyn K), the tryptophan-alkylated byproduct was absent (Figure 2B). The amino acid composition of the crude cleaved peptide was: Glu, 1.03; Leu, 0.99; Thr, 1.99; Phe, 0.98; Pro, 1.00; Gly, 0.96.

Starting with the same Fmoc-PAL-Nle-MBHA-Ⓡ used in the best human gastrin-I synthesis (see Figure 1B), the desired sequence was assembled (protocol of Table II). Cleavage with reagent R, CF_3COOH–thioanisole–EDT–anisole (90:5:3:2), 25 °C, 2 h or 8 h, proceeded in 54 ± 2% yield, based on hydrolysis of the cleaved resin; the hydrolysate of material released into solution *lacked entirely* norleucine. Upon analytical HPLC, the desired peptide corresponded to ≈96% of the total material. The amino acid composition of the crude cleaved peptide was: Glu, 1.06; Leu, 0.94; Thr, 1.75; Phe, 0.95; Pro, 0.96; Gly, 1.03. Application of CF_3COOH–thiophenol (24:1, v/v), 25 °C, 8 h, gave 44% cleavage (compare to the result of Table I line 4). The crude *cleaved* peptide showed two peaks on analytical HPLC, byproduct:desired = 1:2; the hydrolysate of this material *included* norleucine (0.36 with respect to other residues).

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Registry No. **1**, 115109-65-4; **1′**, 115109-66-5; **2**, 500-99-2; **3**, 22080-96-2; **4**, 708-76-9; **5**, 125666-65-1; **6**, 14660-52-7; **7**, 115109-57-4; **7′**, 115109-58-5; **8**, 115109-59-6; **8′**, 115109-60-9; **9**, 115109-61-0; **9′**, 115109-62-1; **10**, 125666-67-3; **10′**, 125666-73-1; **11**, 125666-68-4; **12**, 125666-70-8; **12** (free base), 125666-69-5; **12** (picrate), 125666-74-2; **13**, 84418-43-9; **14**, 115109-56-3; **15**, 125666-71-9; LH-RH, 33515-09-2; H-*p*Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2, 125666-75-3; H-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala-NH_2, 125666-76-4; H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2, 125666-77-5; H-Gly-Asn-Ile-Tyr-Asp-Ile-Ala-Ala-Gln-Val-NH_2, 125666-78-6; human gastrin 1 heptadecapeptide, 10047-33-3.

Supplementary Material Available: Acidolytic cleavage of anchors derived from pure para and ortho isomers of the PAL handle; analytical HPLC's of crude acyl protein (65–74) amide, retro-acyl carrier protein (74–65) amide, and luteinizing hormone-releasing hormone (LH-RH) prepared by solid-phase methodologies of this paper; reversed-phase MPLC purification of human gastrin-I; amino acid analyses of human gastrin-I; general procedures for FABMS; positive and negative ion FABMS of human gastrin-I; calculated isotope distribution of molecular ion, human gastrin-I; NMR and FABMS of desired and tryptophan-alkylated peptides reported in Table I; raw HPLC data used to derive Table I (19 pages). Ordering information is given on any current masthead page.