Synthesis of Highly Tritiated 4-Benzoyl-L-phenylalanine, a Photoactivatable Amino Acid

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Benzoyl-L-phenylalanine (Bpa) is a benzophenone-containing unnatural amino acid that can be incorporated into polypeptides by solid-phase synthetic techniques as a photoactivatable replacement for aromatic or hydrophobic amino acid residues.[1] The expanding use of benzophenone (BP) photophores in photoaffinity labeling can be attributed to three distinct chemical and biochemical advantages.[2,3] First, BPs are chemically more stable than diazoesters, aryl azides, and diazirines. Second, BPs can be manipulated in ambient light and can be activated at 350–360 nm, avoiding protein-damaging wavelengths. Third, BPs react preferentially with unreactive C–H bonds even in the presence of solvent H_2O and bulk nucleophiles. Bpa-containing peptide photoprobes were successfully employed in determining active site domains for protein–protein interactions.[3] However, high specific activity, tritium-labeled Bpa is unknown, and no protocols have been described for the preparation of tritiatable precursors. As a result, radiotracers have been introduced at some other site in the peptide, such as at the Tyr residues[4] using ^{125}I, via the attachment of [^{125}I]-labeled Bolton–Hunter reagent[5] at lysyl N^{ϵ} or N-terminal amine groups or by acylation of nucleophilic OH or NH_2 groups with [^3H]acetic anhydride[6] or [^3H]-propionyl NHS ester. None of these solutions is ideal, since the photophore and radiolabel are then in different regions of the peptide photoprobe.

Photolabeling by BP photoprobes generally leads to chemo- and regioselective covalent modification of the target protein. Introduction of the photophore and the long half-life, safely-handled tritium label into the BP moiety would facilitate identification of the cross-linking sites. Three modes of introduction are possible. First, [^3H]Bpa could be used as a tracer[7] with nonradioactive Bpa and would be added in N-protected form during peptide synthesis. Second, a tritiatable precursor could be incorporated into the synthetic peptide, and the product peptide could be reductively tritiated. Third, the labeled Bpa could be introduced into a protein in a site-specific fashion using missense tRNA methodology.[8] We report herein a facile synthesis of two different multiply ring-brominated precursors that can be used in each of the three labeling approaches.

Catalytic debromination is the most widely used technique for introducing a tritium label into aromatic rings. Two tritiodehalogenation methods were recently described in which tritiated peptides had been made with [^3H]Tyr[9] or Trp.[10] To achieve maximal specific activities (30–120 Ci/mmol), multiply-brominated Bpa derivatives were envisioned as arising from brominated 4-methylbenzophenone precursors. The bromination of aromatic rings containing deactivating carbonyl substituents is usually sluggish with classical brominating reagents. Nonetheless, perbromination of diaryl ketones has been accomplished using dibromoisocyanuric acid (DBI) as the brominating agent in strong acidic media.[11] In our hands, however, use of excess DBI for each position of 4-methylbenzophenone gave a nonabrominated material, but in unacceptably low yield (10–15%) as part of a complex mixture. The desired specific activity Bpa required only two to four bromines per molecule, but attempts to reduce the molar equivalents of DBI gave an unacceptably complex mixture of polybrominated compounds. Thus, a modified strategy was devised in which the BP photophore was assembled by Friedel–Crafts reactions. The single aromatic ring synthons could then be brominated under more controlled circumstances. Acyl and bromine functionalities were placed in the same aromatic ring, since bromines deactivate the ring that is the target of the acylation.

As shown in the upper portion of Scheme 1, *p*-toluic acid was tetrabrominated by DBI[12] in 15–25% isolated yield. In this reaction the stirring of the thick suspension in sulfuric acid is a critical factor; insufficient stirring resulted in incomplete tetrabromination. On the other hand, an extended reaction time reduced the yield and led to an increase in volatile impurities derived from decarboxylation. The acid chloride of tetrabromo acid **1** was generated *in situ* for Friedel–Crafts acylation[13] to give 4-methyl-2,3,5,6-tetrabromobenzophenone (**2a**) in 94% yield. In the alternative approach shown in Scheme 1, commercially available 2,5-dibromobenzoic acid was converted to its acid chloride and allowed to react with toluene under Friedel–Crafts conditions. The 4-methyl-2′-dibromobenzophenone (**2b**, *p*-isomer) was isolated as major product (67%) with a small amount of *o*-isomer (7%), which was removed by flash chromatography. The *p*-orientation was confirmed by ^1H NMR and by reductive debromination to the known 4-methylbenzophenone.

The dibromo-4-methylbenzophenone (**2b**) was brominated on the methyl group by NBS in the presence of AIBN in 71% yield.[21] For the tetrabromo derivative (**2a**) the radical initiation alone was insufficient, and even with extended reflux time, low conversion rates and unacceptable side products were obtained. Therefore,

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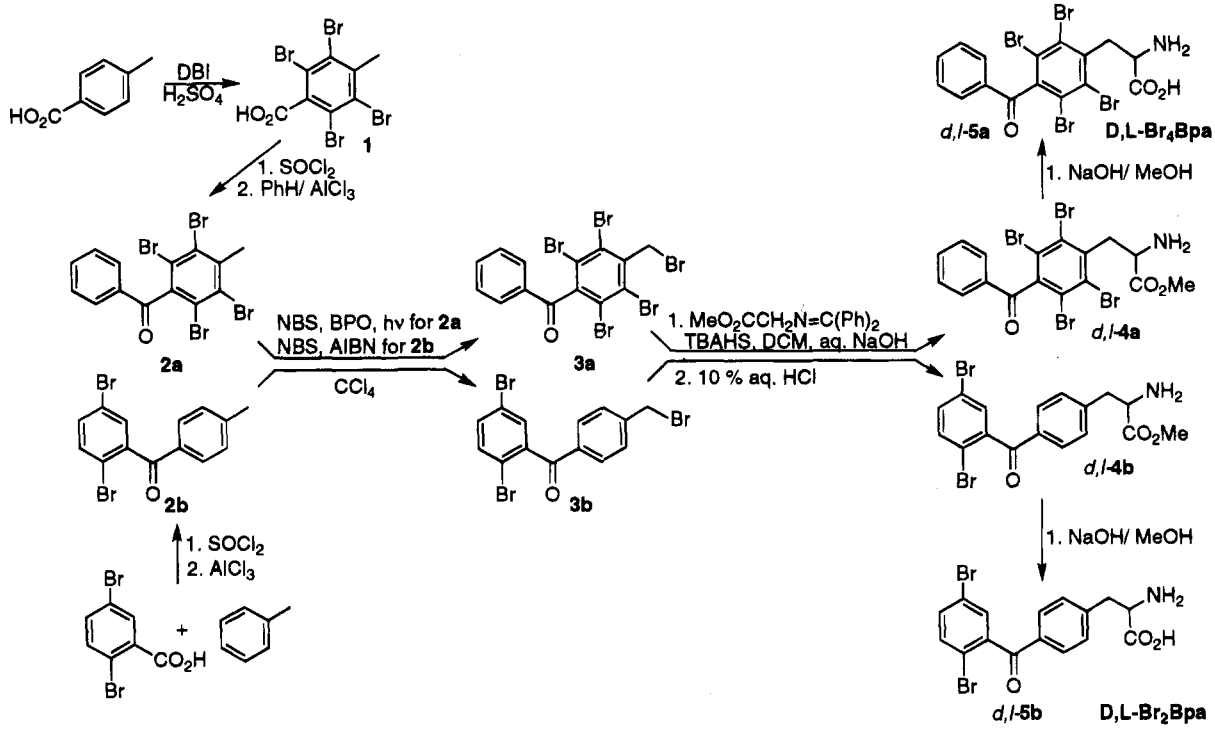
Scheme 1



conditions were changed to employ both photochemically- and radically-initiated (dibenzoyl peroxide) benzylic bromination with NBS. Thus, photolysis using a medium pressure mercury lamp and Pyrex sleeve (290–370 nm) with 2.5 equiv of NBS gave predominantly 4-(bromomethyl)-2,3,5,6-tetrabromobenzophenone (**3a**). It is interesting to note that the BP photophore, which is activated at 350 nm during photoaffinity labeling experiments, is not degraded during irradiation in CCl_4, even though electron-withdrawing groups would be expected to increase the propensity of the excited carbonyl to undergo hydrogen abstraction. Only a small amount of polar impurity (2–3%) was detected in both cases.

The (bromomethyl)benzophenones **3a** and **3b** were used without further purification for the next step. Only the monobrominated benzylic derivatives were able to alkylate the Schiff's base-activated glycine under basic and phase-transfer conditions.[14] The resulting polar BP imine residues could be separated from the nonpolar impurities by flash chromatography and hydrolyzed to give the racemic free amino acid esters (*d,l*-**4a** and *d,l*-**4b**) in 46% and 37% yield, respectively, for three steps. The esters were subjected to saponification yielding the racemic brominated benzoylphenylalanines (*d,l*-**5a** and *d,l*-**5b**).

The large-scale resolution of the racemic photolabile amino acids can be achieved enzymatically. In our initial attempts we used Acylase I for L-selective hydrolysis of racemic *N*-acetylamino acids[15] prepared in 88% yield by a modified Schotten–Baumann procedure (Scheme 2). The very low solubility of the *N*-acetyl-Br_2Bpa (*d,l*-**6**) and Br_2Bpa in aqueous media affected the resolution and isolation giving L-Br_2Bpa in low yield (20%) although in >99% ee. A "robust" protease, Carlsberg Subtilisin, which tolerates 30% acetone, improved the solubility of the Br_2BpaMe in H_2O and showed enzyme-catalyzed ester hydrolysis. However, high ee was only achieved (≥95%) with N-protected (*t*-Boc or Ac) amino acid esters in accordance with previous observations[22] (Table 1). With unprotected Br_2Bpa, the highest ee was obtained at a lower temperature (15 °C; ~85% ee), and reducing the pH from 8.2 to 7.6 did not alter the product distribution (50% ee).

The kinetic resolution can be monitored by TLC. At ~50% conversion the products were separated either by acidic extraction in the case of unprotected residues (L-Br_2Bpa; D-Br_2Bpa-Me) or by flash chromatography for the corresponding *N*-acylated derivatives. Although the enzymatic hydrolysis of the *N*-Boc-derivative (*d,l*-**7b**) is considerably slower, a higher ee value was achieved than with the *N*-acetyl-Br_2Bpa ester (*d,l*-**9**). Moreover, the resulting *N*-Boc-protected L-dibromo-Bpa (*l*-**8**) can be used without further manipulation in either tritiodehalogenation or solid-phase peptide synthesis. Under similar conditions, the *N*-Boc-protected Br_4Bpa was unexpectedly not a substrate for this enzyme.

The ee determination was performed with unprotected L-Br_2Bpa. Deprotection of the *N*-Boc derivative (*l*-**8**) was performed with 50% TFA/CH_2Cl_2 in 98% yield. First, a semiquantitative TLC analysis was employed using Chiralplate that allowed detection of a 2.5% enantiomeric impurity (95% ee). To obtain more accurate enantiomeric purity values, (*R*)-(+)-Mosher's amide (**10**) was prepared by converting the acid to a methyl ester by standard thionyl chloride/methanol esterification, followed by N-acylation with freshly-prepared (*S*)-(+)-Mosher's acid (MTPA) chloride. The enantiomeric purity was determined by integration of the MTPA methoxy resonances by ^1H NMR; analysis of the racemic and resolved MTPA-amide diastereomers showed ~98% enantiomeric purity for the protease resolution on the Boc-protected Br_2Bpa Me-ester.

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Scheme 2

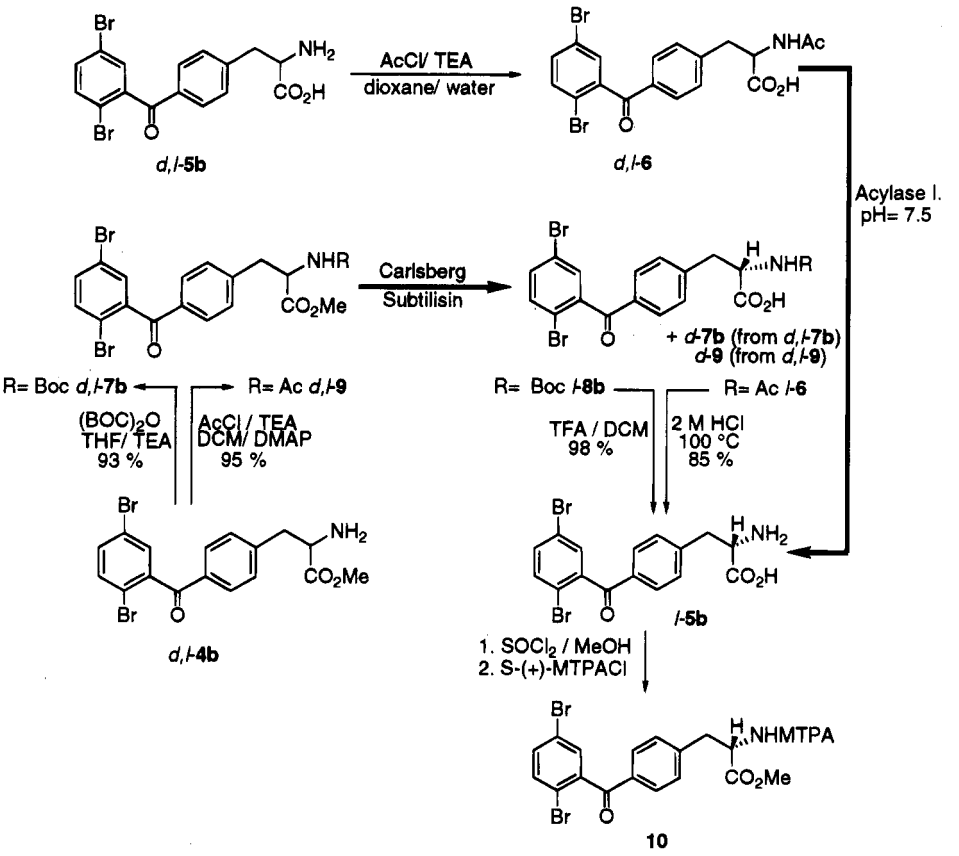
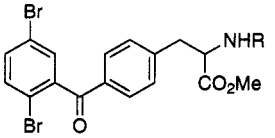
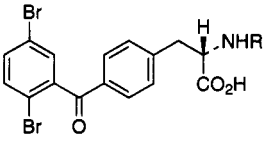


Table 1. Enzymatic Resolution Using Carlsberg Subtiltisin

entry		pH	solvent	T (°C)	time (h)	yield (%)	ee (%)
1	R = H (*d,l*-**4b**)	8.2	water/ acetone	37 °C	2 h	43	50[a]
2	R = H (*d,l*-**4b**)	8.2	water/ acetone	15 °C	3 h	37	~85[b]
3	R = H (*d,l*-**4b**)	7.6	water/ acetone	37 °C	4 h	48	~50[b]
4	R = Ac (*d,l*-**9**)	8.2	water/ acetone	37 °C	2 h	50	~95[b]
5	R = BOC (*d,l*-**7b**)	8.2	water/ acetone	37 °C	8 h	40	98[a]

[a] Ee was determined by ^{1}H NMR spectra of the (*R*)-(+)-Mosher's amide methyl ester. [b] Ee was estimated by separating the enantiomers on Chiralplate.

Selective dehalogenation of racemic Br_4Bpa (*d,l*-**5a**) and Br_2Bpa (*d,l*-**5b**) could be accomplished without reducing the carbonyl groups by using partially-poisoned palladium catalysts and by trapping the released HBr with base. Several recent methods have shown high selectivity in the presence of aromatic carbonyl groups and olefins: Pd/alumina/TEA/THF;[16] 10% Pd/C/methanol/1 N KOH;[17] $Pd(OH)_2$/C/TEA/THF;[18] and PdO/$BaSO_4$/TEA/DMF.[19] The brominated free amino acid precursors were soluble only in a DMF/TEA solvent system. Reductive dehalogenations were performed with both *d,l*-**5a** and *d,l*-**5b**, using either 5% Pd/C or 10% $Pd(OH)_2$/C as catalysts (Scheme 3). The hydrogenation was monitored by reversed-phase HPLC (RP-HPLC). Pearlman's catalyst ($Pd(OH)_2$/C) showed higher selectivity for both dibromo and tetrabromo derivatives; after 30 min, the debromination was complete and exposure to hydrogen gas for an additional 15 min reduction showed little tendency for overreduction. When these hydrogenation conditions were employed for tritiodebromination, [^{3}H]-Bpa was obtained with specific activity 19.5 Ci/mmol from dibromo precursor *d,l*-**5b** and 65.5 Ci/mmol from the tetrabromo precursor *d,l*-**5a**.

For application in peptide photoprobe synthesis, [^{3}H]Bpa, Br_2Bpa, and Br_4Bpa can be employed as their *N*-Boc derivatives. The corresponding amino acids can be protected by the standard protocols (BOC-ON/dioxane/H_2O/TEA) in 85% yield for the brominated precursors and 35% radiochemical yield for the racemic tritiated material.

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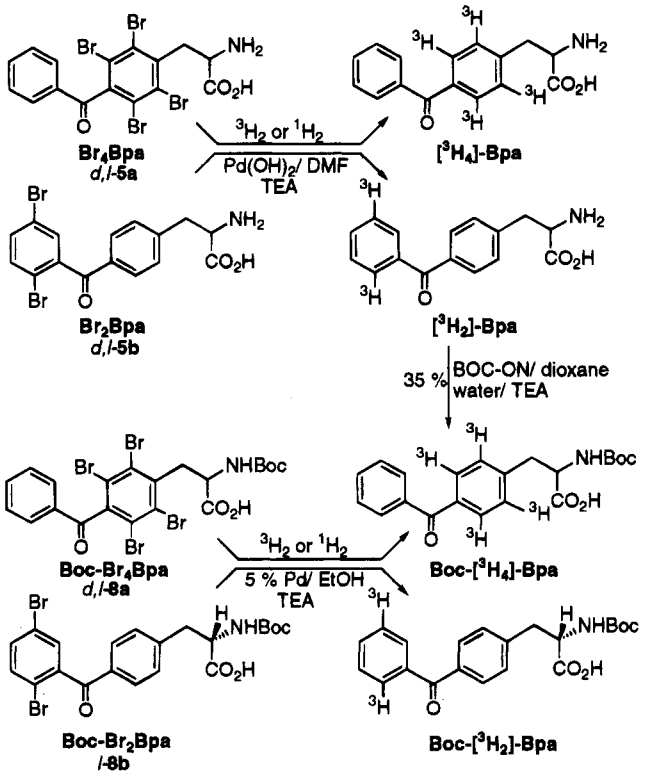
Scheme 3



Alternatively, the reductive debromination can be carried out with equally high selectivity using ethanol/TEA/5% Pd/C on the *N*-Boc-protected amino acids (*d,l*-**8a** and *l*-**8b**); the latter was obtained directly from the enzymatic resolution. This method has the further advantage that the product can be purified simply by silica gel flash chromatography and the microscale Boc derivatization on the tritiated material can be omitted. With this procedure a particularly high specific activity was achieved for the optically-pure [^{3}H]-Boc-Bpa from its dibrominated precursor, 36.6 Ci/mmol, while the racemic tetratritio compound was obtained with a 68.8 Ci/mmol specific activity.

In summary, we have reported a facile synthesis of high specific activity, tritiated 4-benzoyl-L-phenylalanines and two brominated precursors that may be used to prepare photoactivatable polypeptides. Such photoactivatable, radiolabeled peptides will be useful for determining regions of protein–protein interactions in biochemical systems.

Experimental Section

All reagents and solvents were purchased from either Aldrich Chemical Co. or Fisher Scientific and were of reagent grade. RP-HPLC was performed on a RP-300 C_8 column (0.45 cm × 22 cm) using a gradient system equipped with a diode array detector. Benzene and toluene were freshly distilled from sodium benzophenone ketyl. DBI[11] and glycine methyl ester Schiff's base[20] were prepared as described.

2,3,5,6-Tetrabromo-4-methylbenzophenone (2a). The tetrabromination of *p*-toluic acid[12] was performed with minor modifications. An excess (2.5 equiv) of brominating reagent (DBI) was used and dissolved in 95% of the total volume of sulfuric acid. That solution was added in three portions to *p*-toluic acid dissolved in a minimum amount of sulfuric acid. The product was isolated as reported giving tetrabromo-*p*-toluic acid (**1**) in 15–25% yield as a white powder: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.82 (s, 3H, CH_3); mp > 175 Dec. RP-HPLC analysis revealed some percentage of tribrominated-*p*-toluic acid in larger batches due to insufficient stirring; this impurity could not be readily removed and was retained throughout the reaction sequence.

For Friedel–Crafts acylation, the tetrabromo-*p*-toluic acid (0.37 g, 0.82 mmol) was dissolved in 3 mL of thionyl chloride and refluxed for 1 h at 80 °C. Excess reagent was evaporated *in vacuo*, and the residue was dissolved in 1.5 mL of freshly-distilled dry benzene at 45 °C. The solution was cooled to room temperature, and 270 mg of anhydrous $AlCl_3$ was added in five portions. The reaction mixture became dark green, and the temperature was raised to 60 °C. After 2 h the reaction mixture was poured onto a mixture of 14 g of crushed ice and 5 mL of concentrated hydrochloric acid and stirred for 30 min. The aqueous suspension was extracted twice with 10 mL of ether. The ethereal extracts were washed with saturated $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and concentrated. The crude product was purified by flash chromatography over SiO_2 (solvent: 30% CH_2Cl_2/hexane) to give **2a** as a white powder (0.4 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.82 (2H, d); 7.64 (1H, t,); 7.51 (2H, t); 2.9 (3H, s). Mp: 173–175 °C. Anal. Calcd for $C_{14}H_8OBr_4$: C, 32.85; H, 1.58; Br, 62.45. Found: C, 33.02; H, 1.66; Br, 62.36.

2′,5′-Dibromo-4-methylbenzophenone (2b). The Friedel–Crafts acylation of toluene by 2,5-dibromobenzoic acid chloride was carried out as described for **2a**. The corresponding acid chloride was formed by refluxing 13 mL of thionyl chloride and 950 mg (3.35 mmol) of 2,5-dibromobenzoic acid. Then, the acylation was performed with 4.5 mL of freshly-distilled toluene and with 1.09 g (8.35 mmol) of anhydrous $AlCl_3$. The isomers were separated by silica gel chromatography (solvent: 25% CH_2Cl_2/hexane) to give 770 mg (67%) of pure **2b** as a reddish white powder accompanied by some mixed fractions (50 mg) and pure *ortho* isomer (65 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.70; 7.45–7.55; 7.28; 2.42. ^{13}C NMR (63 MHz, $CDCl_3$) δ: 193.8; 145.3; 142.6; 134.5; 134.0; 133.0; 131.6; 130.4; 129.5; 121.3; 118.15; 21.9. Mp: 97 °C. Anal. Calcd for $C_{14}H_{10}OBr_2$: C, 47.49; H, 2.85. Found: C, 47.70; H, 3.03.

Methyl 4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-4a). The benzylic bromination was carried out in a sealed tube containing **2a** (216 mg, 0.42 mmol), 1.6 mL of dry CCl_4, NBS (200 mg, 1.12 mmol), and 6 mg of dibenzoyl peroxide under argon. The reaction mixture was irradiated with a medium pressure mercury lamp for 2.5 h at 80 °C. After the mixture was cooled, succinimide was removed by filtration and washed twice with 2 mL of CCl_4. The combined filtrates were washed with 4 mL of saturated aqueous $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and evaporated to give a yellow solid (240 mg) that was identified as >90% of 4-(bromomethyl)-2,3,5,6-tetrabromobenzophenone (**3a**). ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.80 (2H, d, phenyl C-2,6); 7.65 (1H, t); 7.52 (2H, t); 5.13 (2H, s, CH_3). IR (Nujol, cm^{-1}): 1677 (C=O).

Crude **3a** was used to alkylate glycine methyl ester Schiff's base under phase-transfer conditions. To a solution of glycine methyl ester Schiff's base (94 mg, 0.371 mmol), 4-(bromomethyl)-2,3,5,6-tetrabromobenzophenone (**3a**) (240 mg, 0.405 mmol), and tetrabutylammonium hydrogen sulfate (TBAH) (127 mg, 0.371 mmol) in 1 mL CH_2Cl_2 was added 1 mL of 10% aqueous NaOH. The two-phase mixture was stirred vigorously, and the organic layer gradually became greenish brown. After 1 h, the phases were separated and the aqueous phase washed twice with 2.5 mL of CH_2Cl_2. The combined organic layers were evaporated, dissolved in EA (25 mL), and washed with 5 mL of H_2O and brine. After being dried over $MgSO_4$ and evaporated, the mixture was chromatographed on silica gel (solvent: 75% CH_2Cl_2/hexane) to give 188 mg of methyl *N*-(diphenylmethylidene)-4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine, which slowly lost benzophenone upon storage. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 6.80–7.80 (15 H, m); 4.90 (1H, d); 4.22 (1H, dd); 3.85 (3H, s); 3.80 (1H, m).

Hydrolysis of the benzophenone imine was accomplished in 0.3 mL of THF and 0.9 mL of 5% HCl. After the mixture was stirred for 2 h at room temperature, saturated $NaHCO_3$ was added and the basic mixture was extracted with EA (3 × 5 mL). The combined organic extracts were washed with H_2O and brine (3 mL each), dried, and evaporated. The crude product was

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chromatographed on silica gel (solvent: 75% EA/H) and gave methyl 4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**4a**) as a white powder (130 mg, 46% from **2a**). ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.84 (2H, d); 7.62 (1H, t); 7.53 (2H, t); 4.0 (1H, t, C*H*); 3.78 (3H, s); 3.74 (2H, m). ^{13}C NMR (63 MHz, $CDCl_3$) δ: 192.5; 175.2; 142.6; 134.5; 130.2; 129.9; 129.2; 128.9; 122.4; 54.2; 53.8; 45.0. Mp: 147–150 °C. Anal. Calcd for $C_{17}H_{13}O_3Br_4$: C, 34.09; H 2.18. Found: C, 34.51; H, 2.23.

Methyl 4-Benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-4b). The benzylic bromination of **2b** was carried out according to Itoh[21] in 71% yield. The crude material was partially purified by flash chromatography (5% EA/H), and the 4-(bromomethyl)-2′,5′-dibromobenzophenone (^{1}H NMR: δ 4.55 (2H, s, benzylic CH_2)) was used to alkylate the glycine Schiff's base. The phase-transfer reaction was carried out in a CH_2Cl_2–10% aqueous NaOH (5/5 mL) two-phase system containing glycine methyl ester Schiff's base (0.975 g, 3.85 mmol), 4-(bromomethyl)-2′,5′-dibromobenzophenone (**3b**, 2.0 g, 4.24 mmol, ~90%), and TBAH (1.30 g, 3.85 mmol). The more polar alkylated product was separated by silica gel column chromatography (solvent: 75% CH_2Cl_2/hexane; then CH_2Cl_2) resulting in methyl *N*-(diphenylmethylidene)-4-benzoyl-2′,5′-dibromophenylalanine (1.94 g; 63%, oil). ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.62 (2H, d); 7.55 (2H, d); 7.49-7.24 (9H, m); 7.14 (2H, d); 6.67 (2H, d); 4.30 (1H, dd); 3.73 (3H, s); 3.33 (2H, m).

The benzophenone–imino acid ester was stirred in a mixture of 2 mL of THF and 6 mL of 5% HCl for 2 h. The deprotected methyl 4-benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-**4b**) was purified on silica gel as described for *d,l*-**4a** to give 270 mg as a white, oily powder in 36% yield from **2b**. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.74 (2H, d); 7.42–7.54 (3H, m); 7.32 (2H, d); 3.77 (1H, dd); 3.70 (3H, s); 3.02 (2H, 2 × dd). ^{13}C NMR: (63 MHz, $CDCl_3$) δ: 193.7; 175.1; 144.8; 143.0; 134.6; 134.1; 131.6; 130.6; 130.5; 129.8; 121.6; 117.7; 55.5; 52.1; 41.0. Anal. Calcd for $C_{17}H_{15}NO_3Br_2$: C, 46.28; H, 3.43; Br, 36.23. Found: C, 46.39; H, 3.51; Br, 36.19.

4-Benzoyl-2′,5′-dibromo-D,L-phenylalanine Hydrochloride (*d,l*-5b). To a solution of methyl 4-benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-**4b**) (640 mg, 0.082 mmol) in 7.50 mL of methanol was added 112 mg of NaOH in 1.7 mL of H_2O, and the resulting suspension was stirred for 10 h at room temperature and gradually clarified. The reaction mixture was acidified by 1 M HCl, and the precipitate was filtered. The filter cake was washed with EA and dried *in vacuo* overnight giving a white powder (550 mg). ^{1}H NMR (300 MHz, d_6-DMSO) δ: 7.65–7.78 (5H, m); 7.42–7.50 (2H, d); 3.6 (1H, m, C*H*); 2.97 (2H, 2 × dd, CH_2). Anal. Calcd for $C_{16}H_{14}NO_3Br_2Cl$: C, 41.25; H, 3.04; Br, 34.47. Found: C, 41.26; H, 3.26; Br, 34.21. Purity by RP-HPLC: >99% (Aquapore RP-300 C_8, mobile phase: gradient with A: 0.1% TFA/H_2O and B: 95% CH_3CN/H_2O; retention time 31 min).

4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-5a). Methyl 4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**4a**) was similarly hydrolyzed in methanolic NaOH as described for *d,l*-**5b** in 87% yield. ^{1}H NMR (300 MHz, d_6-DMSO) δ: 7.75 (2H, d); 7.70 (1H, t); 7.58 (2H, t); 3.78 (1H, t); 3.58 (2H, m). FAB MS: calcd for $C_{16}H_{11}Br_4NO_3$ 584.88, found 585. RP-HPLC: retention time 31.6 min (Aquapore RP-300 C_8, mobile phase: gradient with A: 0.1% TFA/H_2O and B: 95% CH_3CN/H_2O; occasionally tribrominated product was also detected: retention time 29.7 min).

***N*-(*tert*-Butoxycarbonyl)-4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-8a).** To a suspension of 4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**5a**) (20 mg; 34 μmol) in 0.3 mL of 50% aqueous dioxane was added 12 μL of triethylamine (85 μmol) and 10 mg of BOC-ON ([(*tert*-butoxycarbonyl)oxy]imino)-2-phenylacetonitrile; 40 μmol). After 8 h of stirring at room temperature, the suspension turned into a clear solution, which was then concentrated by a stream of nitrogen. The residue was dissolved in 0.5 mL of EA, 0.5 mL of water was added, the aqueous phase was acidified (10% citric acid, pH = 2.0), and the EA layer was separated. After two extractions with EA, the combined extracts were washed (H_2O, brine), dried ($MgSO_4$), and evaporated under nitrogen. The crude product was purified by flash chromatography eluting with $CHCl_3$/EA/HOAc 5:2:0.1 or $CHCl_3$/MeOH 10:1 giving 19.8 mg (85%) of *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**8a**). ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.84 (2H, d); 7.65 (1H, t); 7.51 (2H, d); 5.35 (1H, d); 4.90 (1H, m); 3.80–3.85 (2H, m); 1.39 (9H, s). FAB MS: calcd for $C_{21}H_{19}Br_4NO_5$ 685.0, found 685.

N-Boc-[^{3}H$_4$]-Bpa was similarly prepared from [^{3}H$_4$]-Bpa (S. A. 65.5 Ci/mmol) on a 5 mCi scale. After flash chromatography, 1.75 mCi was recovered as a radiochemically-pure compound (35%).

The racemic *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-**8b**) was similarly prepared in 90% yield; however, its characterization will be presented as the optically-pure material (*l*-**8b**) derived from the enzymokinetic resolution.

Enzymatic Resolution of 4-Benzoyl-2′,5′-dibromo-D,L-phenylalanine. a. Acylase I. *N*-Acetyl-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-**6**) was prepared by a modified Schotten–Baumann procedure. To an ice-cold solution of 4-benzoyl-2′,5′-dibromo-D,L-phenylalanine hydrochloride (106 mg, 0.230 mmol) in 0.8 mL of H_2O, 0.8 mL of 1,4-dioxane, and 0.15 mL of TEA was added acetyl chloride (23 μL, 0.35 mmol). The greenish solution was stirred for 24 h at 5 °C, and then an additional amount of acetylating reagent was added (10 μL) and stirred for 12 h at room temperature. The reaction mixture was acidified with 1 N HCl and diluted with 5 mL of H_2O. The solution was extracted with $CHCl_3$ (3 × 1 mL), and the organic phase was washed with H_2O and brine. The organic layer was dried on a $MgSO_4$ pipette column and evaporated, resulting in a yellow powder (97 mg, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.75 (2H, d); 7.45–7.55 (3H, m); 7.28 (2H, d); 5.9 (1H, d, N*H*); 4.9 (1H, m, C*H*); 3.25 (2H, 2 × dd), 2.0 (3H, s).

To a suspension of *N*-acetyl-Br$_2$Bpa (97 mg, 0.207 mmol) in 10 mL of H_2O (pH = 7.5 adjusted with 4.7 M NH_4OH) containing 0.2 mL of 2-propanol was added 2 mg of aspergillus acylase I and 2 mg of $CoCl_2$. The suspension was stirred at 37–40 °C for 3 days. TLC analysis revealed that the enzymatic hydrolysis stopped at 40% conversion and did not change when additional portions of enzyme (2 mg) were added. The enantiomerically-pure L-Bpa (12 mg; >99% ee) was isolated according to Pirrung's procedure,[14] and a second crop was obtained by repeated ethanolic extraction under basic conditions (10 mg).

b. Carlsberg Subtilisin. The *N*-acetyl-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine methyl ester (*d,l*-**9**) was prepared using 1.2 equiv of acetyl chloride/2.5 equiv of TEA in CH_2Cl_2 in the presence of a trace of (dimethylamino)pyridine (DMAP) in 95% yield. For *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine methyl ester (*d,l*-**7b**), Lankiewicz's procedure was employed[23] with minor modifications: methyl 4-benzoyl-2′,5′-dibromo-D,L-phenylalanine (315 mg; 0.72 mmol) was dissolved in 1.5 mL of dry THF, and 0.11 mL of TEA was added at 0 °C. The mixture was treated with 0.18 mL (0.77 mmol) of di-*tert*-butyl dicarbonate. After being stirred for 2 h, the reaction mixture was concentrated *in vacuo* and the residue taken up in 10 mL of ethyl acetate. The organic phase was washed (H_2O, 3 mL, and brine, 3 mL), dried ($MgSO_4$), and evaporated. The product was purified by silica gel chromatography (33% EA/H) giving 315 mg of *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine methyl ester in 93% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.72 (2H, d); 7.43–7.52 (3H, m); 7.25 (2H, d); 5.0 (1H, d); 4.63 (1H, m); 3.71 (3H, s,); 3.14 (2H, 2 × dd); 1.40 (9H, s). Mp: 111–112 °C.

The purified *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2′,5′-dibromo-D,L-phenylalanine methyl ester (100 mg, 0.185 mmol) was dissolved in 0.3 mL of acetone, and 5 mg of Carlsberg Subtilisin in 1 mL of sodium bicarbonate buffer (0.2 M; pH = 8.16) was added. The clear solution was stirred vigorously at 37 °C, and soon the hydrolyzed product precipitated. The progress of the hydrolysis was monitored by TLC (10% $CH_3OH/CHCl_3$) until 50% of the substrate was hydrolyzed (~8 h). The reaction was quenched by addition of 1 N HCl (1 mL), and the mixture was extracted with EA (3 × 1.5 mL). The organic extracts were evaporated, and the products were separated by silica gel column chromatography (8% $CH_3OH/CHCl_3$) to give 39 mg of Boc-L-Br$_2$-

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Bpa (40%). ^{1}H NMR (250 MHz, $CDCl_3$): 7.73 (2H, d); 7.43-7.52 (3H, m); 7.25 (2H, d); 5.05 (1H, d); 4.62 (1H, m); 3.25 and 3.22 (2H, 2 × dd); 1.39 (9H, s). ^{13}C NMR (63 MHz, $CDCl_3$): δ 193.8, 173.3, 170.2, 143.1, 142.3, 135.6, 134.3, 134.6, 131.6, 130.5, 129.8, 121.6, 118.1, 80.5, 54.2, 40.9, 28.2. FAB MS: calcd for $C_{21}H_{22}Br_2NO_5$ 528.22, found 528.0. Unreacted Boc-Br_2-D-BpaMe (45 mg) was also isolated during the chromatographic separation.

The ee determination was made first on a Chiralplate using unprotected L-Br_2Bpa. The *N*-acetyl group was removed in refluxing 2 M HCl for 6 h (85%), and the *N*-*t*-Boc-group was cleaved with 50% TFA/CH_2Cl_2 at 0 °C for 30 min in almost quantitative yield (98%). The volatile materials were evaporated, and the residue was diluted with ether and triturated. The resulting precipitate was centrifuged at 0 °C and dried after removing the supernatant solvent. For semiquantitative analysis, 2.5 μL was applied from a 1 mg/1 mL methanolic solution onto the Chiralplate, which was eluted with $CH_3CN/CH_3OH/H_2O$ (4:1:1). The low solubility of Br_2Bpa in solvents allowed a detection limit of ~2.5%, corresponding to ≥95% ee. Visualization was achieved by spraying with 2% ninhydrin in acetone.

For more accurate ee determination, a small amount of D,L-Br_2-Bpa and L-Br_2-Bpa were converted into the corresponding methyl esters by stirring with 1.2 equiv of $SOCl_2$ in dry CH_3OH. The methyl esters were N-acylated with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher's acid chloride) in the presence of pyridine (36 h at 40 °C). The resulting racemic and L-4-benzoyl-2′,5′-dibromophenylalanine methyl ester (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetamides were analyzed by 300 and 600 MHz ^{1}H NMR.

Hydrogenation of 4-Benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-5b) and 4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-5a). The hydrogenation was carried out in a round-bottom flask with a side arm. The brominated amino acids (2−3 mg) were suspended in 1 mL of DMF containing 0.1 mL of TEA, and 2 mg of 20% $Pd(OH)_2$/C catalyst was added. A greenish color gradually developed during the reduction. At 20, 40, and 60 min, aliquots were taken for TLC and HPLC analysis. After 60 min, the catalyst was filtered on a Celite bed and washed with 0.5 mL of DMF. The solution was evaporated at 55 °C *in vacuo*. HPLC showed that after 20 min the debromination was complete; further hydrogenation did not cause significant reduction of the carbonyl group. Using the HPLC conditions described above, retention times were 24.5 min (Bpa), 31.0 min (Br_2-Bpa), and 31.6 min (Br_4Bpa). Coinjection with Bpa was used to identify the debrominated product.

Tritiation of 4-Benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-5b) and 4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-5a). Reduction using carrier-free tritium gas was performed at DuPont-New England Nuclear. The procedure for both *p*-benzoyl-2′,5′-dibromo-D,L-phenylalanine (*d,l*-**5b**) and *p*-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**5a**) was the same. A mixture of *d,l*-**5a** or *d,l*-**5b** (10 mg) and 20% $Pd(OH)_2$/C (10 mg) suspended in 5 mL of DMF containing 0.5 mL of TEA was stirred under an atmosphere of carrier-free tritium gas for 90 min. Labile tritium was removed and the mixture filtered through a bed of Celite (1 in. × 0.25 in.), washing with 20 mL of MeOH. The products were purified (>97%) by preparative TLC (Whatman LK5 silica; $CHCl_3/MeOH/H_2O$, 65:25:4). The specific activities of the products were 19.5 Ci/mmol for *d,l*-**5b** and 65.5 Ci/mmol for *d,l*-**5a**, as determined by MS.

Hydrogenation of Boc-Protected 4-Benzoyl-2′,5′-dibromo-L-phenylalanine (*l*-8b) and 4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-8a). To a solution of *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2′,5′-L-phenylalanine (5 mg; *l*-**8b**) in 1 mL of absolute ethanol and 0.1 mL of dry TEA was added 2 mg of 5% Pd/C catalyst. The resulting solution was hydrogenated for 90 min. The reduction was monitored by TLC (mobile phase: $CHCl_3$/EA/acetic acid, 100:12:0.5). The catalyst was filtered through Celite; the filtrate was concentrated. The residue was taken up in 0.1 M HCl (1 mL) and extracted with EA (3 × 1 mL). The organic extracts were washed with H_2O and brine, dried on $MgSO_4$, and concentrated. ^{1}H NMR spectra showed complete hydrodebromination on the ring without reduction on the carbonyl group. Under analogous conditions, the corresponding tetrabromo derivative was reduced for 2 h with similarly high selectivity (4.5 mg *N*-(*tert*-butoxycarbonyl)-4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine; 1.5 mg of 5% Pd/C; 0.8 mL of absolute ethanol; 0.08 mL of TEA).

Tritiation of Boc-Protected 4-Benzoyl-2′,5′-dibromo-L-phenylalanine and 4-Benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine. Hydrogenation using carrier-free tritium gas was performed by DuPont-New England Nuclear. The procedure for both *N*-*t*-Boc-4-benzoyl-2′,5′-dibromo-L-phenylalanine (*l*-**8b**) and *N*-*t*-Boc-4-benzoyl-2,3,5,6-tetrabromo-D,L-phenylalanine (*d,l*-**8a**) was the same. A mixture of *l*-**8b** (10 mg, 0.019 mmol) and 5% Pd/C (4 mg) suspended in 2 mL of EtOH containing 0.2 mL of TEA, or *d,l*-**8a** (5 mg, 0.007 mmol) and 5% Pd/C (1.7 mg) suspended in 1 mL of EtOH containing 0.1 mL of TEA, was stirred under an atmosphere of carrier-free tritium gas for 90 min. Labile tritium was removed and the mixture filtered through a bed of Celite (1 in. × 0.25 in.) washing with 10 mL of MeOH. The crude radioactive yields were 600 mCi from *l*-**8b** and 500 mCi from *d,l*-**8a**. The products were purified (>97%) by HPLC using a Zorbax C8 column with CH_3CN/0.1% TFA (40:60) as the solvent. The specific activities of the products were 36.6 Ci/mmol for [$^3H_2$]-N-Boc-Bpa and 68.8 Ci/mmol for [$^3H_4$]-*N*-Boc-Bpa, as determined by UV spectrometry using ϵ_{265} = 26 568 cm^{-1} M^{-1}.

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