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Synthesis of Biosynthetic Precursors of Chromophores of Red Fluorescent Proteins

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Abstract—A method for the synthesis of 5-arylidene-3,5-dihydro-4*H*-imidazol-4-ones corresponding to the chromophore of the green fluorescent protein (GFP) with acylaminoalkyl substituents at position 2 of the imidazolone core has been developed. These biomimetic model compounds are the precursors of the chromophores of red fluorescent proteins. The method is based on the masking of the dehydrotyrosine fragment of target compounds by the β-hydroxytyrosine moiety. The key stages of the synthesis include the condensation of β-hydroxytyrosine with the appropriate *N*-acetylamino acid, the unmasking of dehydrotyrosine by *O*-acylation with subsequent elimination, and the cyclization of the resulting 3-acylaminocinnamic acid derivatives in basic medium.

Keywords: chromophore, imidazolone, green fluorescent protein, GFP, DsRed

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INTRODUCTION

The popularity of using the green fluorescent protein and other fluorescent proteins as genetically encoded labels largely determines a close interest in studies of the natural diversity, structure, and biochemical and biophysical properties of the proteins of this family. As distinct from other proteins, GFP-like proteins are capable of forming the chromophore group independently, without the participation of external cofactors and enzymes, by the posttranslational modification of their own amino acid residues. It has been established that the chromophores of the GFP family have a common structural core, 5-(4-hydroxybenzylidene)-3,5-dihydro-4*H*-imidazol-4-one. This fragment is formed by cyclization, dehydration, and oxidation (each of the three stages, in turn, is divided into elementary stages whose nature has not been precisely determined) of three neighboring amino acids at positions 65–67 (numbering is that of GFP from the jellyfish *Aequorea victoria*) with the involvement of molecular oxygen. If no further transformations occur, a GFP is formed. However, the amino acid residue at position 65 can undergo further modification, with the result that the conjugated system of π-bonds increases, and a chromophore with absorption at longer wavelengths is formed (red, yellow, and purple proteins). The nature and mechanism of these additional modifications are poorly understood presently.

Earlier, methods for the chemical synthesis of model compounds, the analogues of the chromophore of the GFP, and some other fluorescent proteins, have been developed [1]. The synthesis of model chromophores made it possible to obtain independent proof of the chemical structure of natural chromophores and clarify the details of their spectral properties and the effect of various environmental factors on them [2–5].

The present study deals with the synthesis of 5-(4-hydroxybenzylidene)-3,5-dihydro-4*H*-imidazol-4-ones containing an acylaminoalkyl substituent at position 2 of the imidazolone core. These compounds are the biomimetic precursors of red chromophores and will be further used for studying the mechanism of the oxidation of the C^{α}-N bond of the residue at position 65, which occurs in many red fluorescent proteins and chromoproteins.

The known methods of the synthesis of substituted imidazolones involve: (a) the cyclization of dehydrotyrosine derivatives by the action of bases [6], (b) the reaction of imidates [7, 8] and amidines [9] with appropriate 1,2-dinucleophiles, (c) the cyclization of α-azidoimides by the action of triphenylphosphine [10], and (d) the cross-coupling of boronic acids with thioimidazolones [11].

The necessity of introducing the α-acylamino group at position 2 of imidazolone substantially limits the set of available synthetic methods. Methods (c) and (d) cannot

Abbreviations: AIBN, asobisisobutyronitrile; Bn, benzyl; DsRed, the red fluorescent protein of the coral polyp *Discosoma*; ESI, electrospray ionization; GFP, green fluorescent protein; HOBt, *N*-hydroxybezotriazole; MALDI, matrix-assisted laser desorption ionization; NBS, *N*-bromosuccinimide; PMB, *p*-methoxybenzyl; TBS, *tert*-butyldimethylsilyl.

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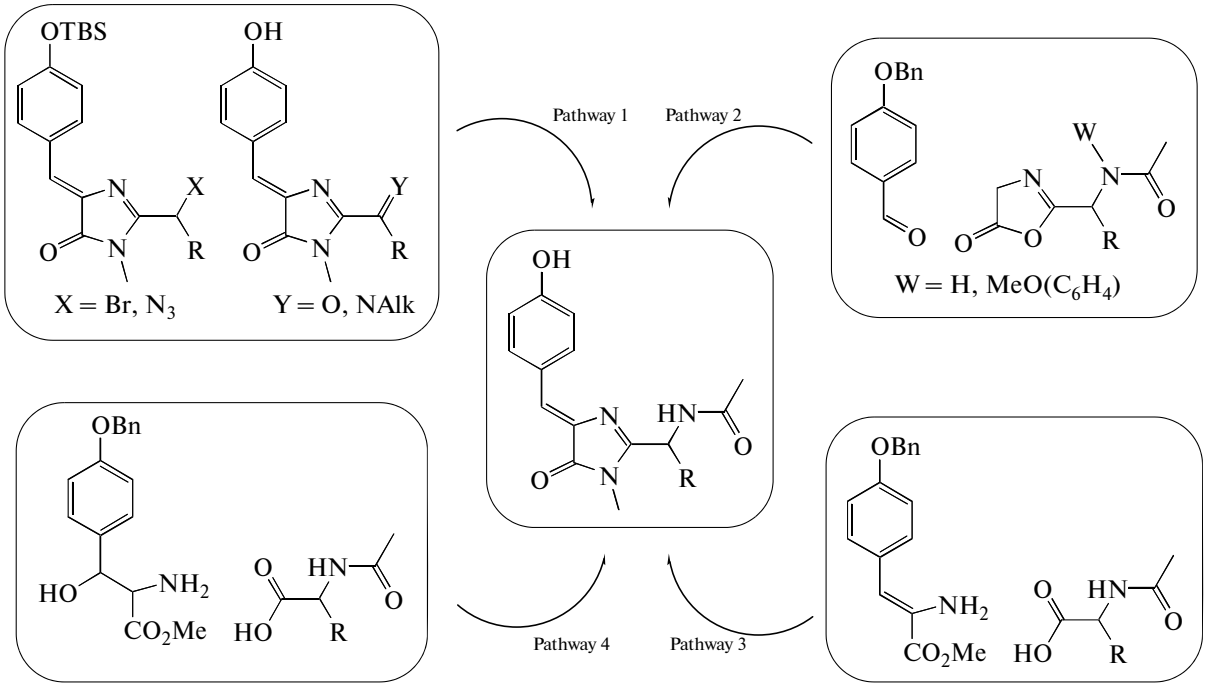
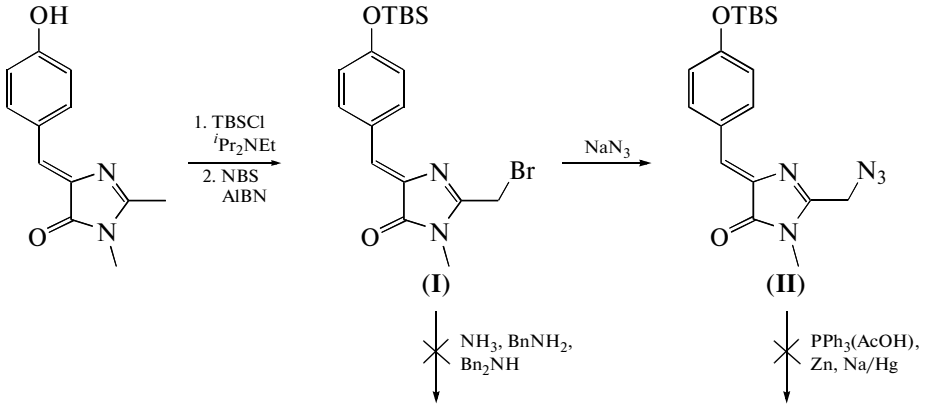
be applied in this case, since there are no approaches to the synthesis of the corresponding α-acylamino-substituted imides and boronic acids. Rigorous conditions of the synthesis of iminoesters and amidines [method (b)] are applicable only to the most inert amino acid residues; in addition, the cyclization of alkyl-substituted amidines is characterized by extremely low yields (<10%) [7]. Based on the aforementioned, it was decided to use the cyclization of dehydrotyrosine derivatives [method (a)] as a key stage of the assembly of the imidazolone core. Below four successive attempts of synthesizing the target compounds are discussed (Scheme 1).



Scheme 1. Four approaches to the synthesis of 3-methyl-2-(α-acetylamino)alkyl-5-arylidene-3,5-dihydro-4*H*-imidazol-4-ones: functionalization of the α-position of the heterocycle (1); assembly of the heterocyclic core using oxazolones (2), dehydrotyrosine (3), and hydroxytyrosine.



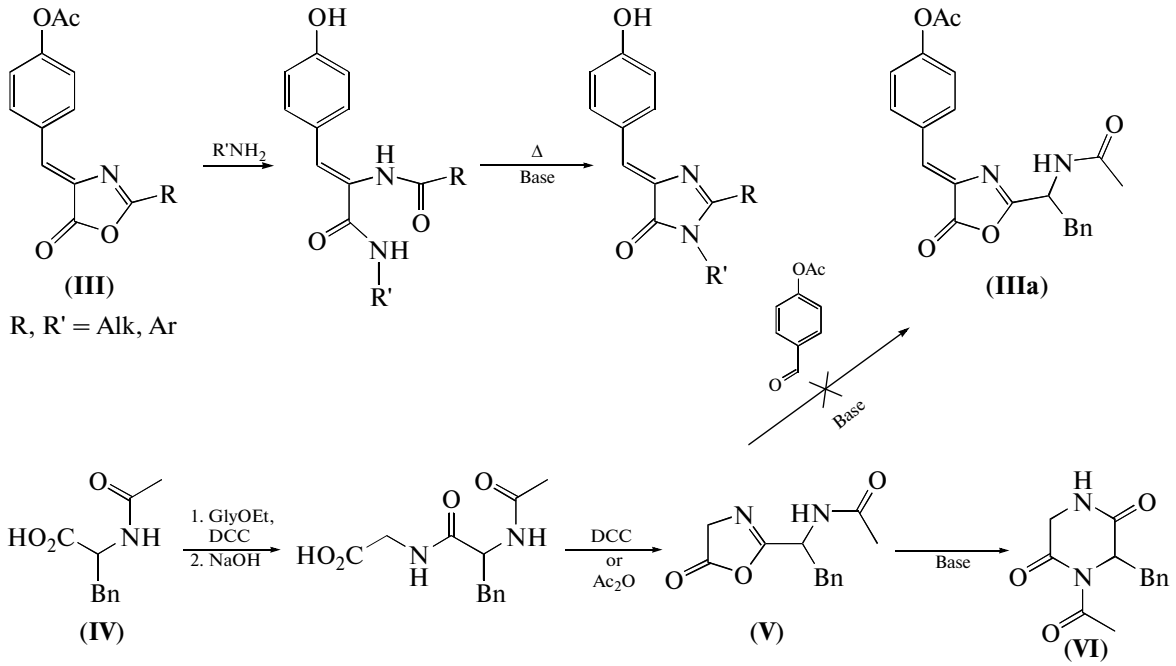
Scheme 2. Functionalization of the α-position of 2,3-dimethyl-5-arylidene-3,5-dihydro-4*H*-imidazol-4-one.

RESULTS AND DISCUSSION

Synthetic approach no. 1. The most adequate approach to the synthesis of α-substituted heterocycles, including imidazolones, is the functionalization of the α-position of the heterocycle. We have previously developed in our laboratory a method for synthesizing α-keto- [3] and α-alkylimino imidazolones [5]. However, numerous attempts to reduce the double C=N bond and synthesize α-acylimino derivatives based on the compounds obtained failed. In the present work, *O*-silylated 2-bromomethylimidazolone (**I**) was successfully synthesized (Scheme 2); however, during

subsequent reactions with amines, ammonium, and acetamide, a complete degradation of the imidazolone cycle occurred. We also failed to convert 2-azidomethyl imidazolone (**II**) to amino derivatives through the reduction by triphenylphosphine (probably due to a high nucleophilicity of the intermediate ilide) and other reductants.

Synthetic approach no. 2. The most universal pathway of de novo assembling the heterocyclic core of 5-arylidene-3,5-dihydro-4*H*-imidazol-4-ones involves the use of 4-arylidene-4,5-dihydrooxazol-4,5-ones (**III**) (Scheme 3). Compounds (**III**) can be obtained through the azlactonization of appropriate *N*-acylglycines and subsequent condensation with 4-hydroxybenzaldehyde [3, 12, 13]. Despite numerous attempts to condense α-acylamino-substituted azlactones with aldehydes under the conditions of base or acid catalysis, we were unable to synthesize target oxazolones of type (**III**). The main process in the case of *N*-acetylphenylalanine (**IV**) is the isomerization [14] of saturated azlactone (**V**) to *N*-acetyldiketopiperazine (**VI**) (Scheme 3).

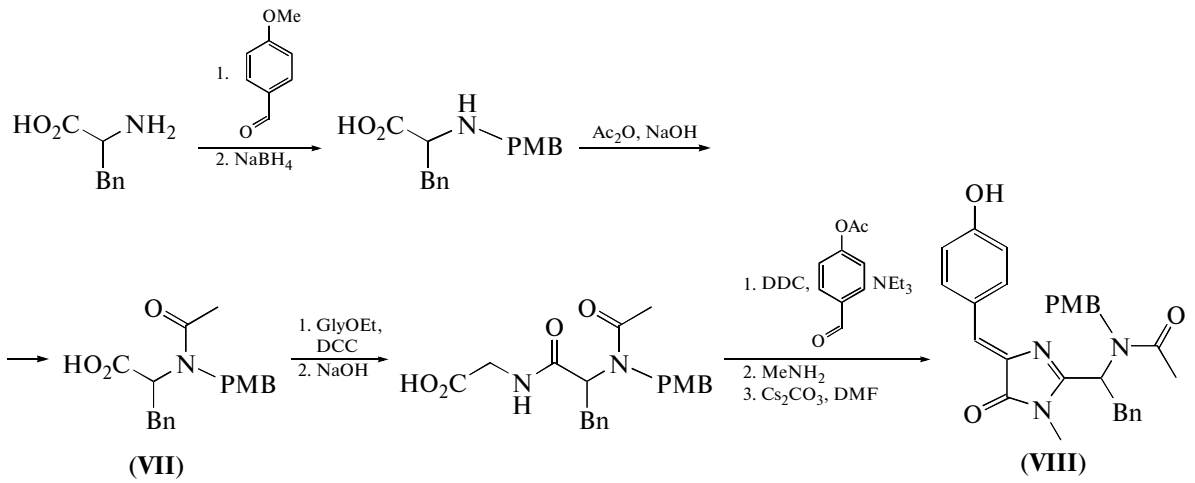


Scheme 3. General strategy of the synthesis of 5-arylidene-3,5-dihydro-4*H*-imidazol-4-ones based on oxazolones (**III**). Synthesis with the use of *N*-acetylphenylalanine.

The problem of eliminating the isomerization at the condensation stage can be solved by the introduction of a protecting group at the amide atom of nitrogen of acetyl amino acid. The 4-methoxybenzyl group, which provides the sufficient flexibility in the choice of deprotection conditions (reduction, oxidation, and acidolysis), served as the protecting group. The corresponding *N*-PMB-*N*-acetylphenylalanine (**VII**) was successfully synthesized and converted to imidazolone (**VIII**) with the residue of *N*-protected phenylalanine (Scheme 4).

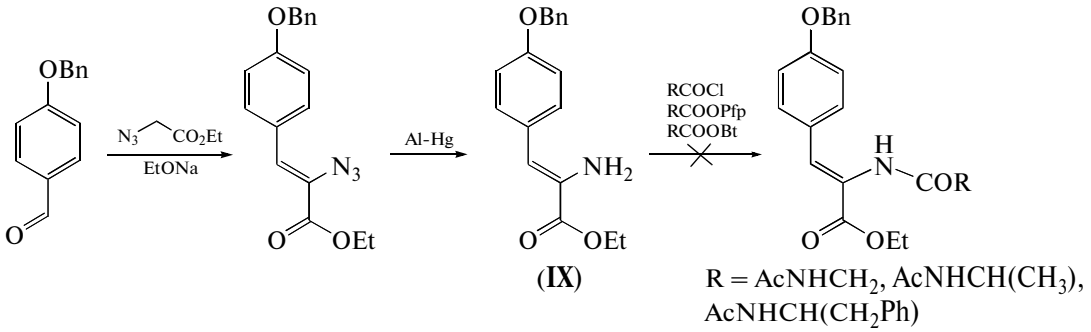
It was found in subsequent experiments that the *para*-methoxybenzyl group in compound (**VIII**) cannot be selectively removed during catalytic hydration and reduction by metals and other reagents. Simultaneously with the deprotection, the hydration of the C=C double bond of the imidazolone core occurs. The reoxidation of dihydroimidazolone by the method described in [15] occurs with a very low yield and cannot be considered as the preparative reaction. The oxidative deprotection of PMB amide (**VIII**) by the action of ceric ammonium nitrate leads to the degradation of the imidazolone core, including the case that there is a methyl group on the oxygen atom of the phenol fragment.

As known, the methoxybenzyl group can be removed in some cases by strong acids (among other things, in the presence of the scavenging nucleophiles: thioanisole, pentamethylbenzene, etc.). In our case, the acidolysis of the methoxybenzylamide fragment occurred only by the action of such a strong acid as trifluoromethanesulfonic acid in a non-basic solvent (for example, dichloromethane) with a low yield (<20%). After the addition of nucleophiles, the yield of the target product decreased to 5% and less. Unexpectedly, the severe deprotection conditions appeared to be inappropriate for other amino acid residues (alanine and glycine). A complex mixture of degradation products formed from the corresponding chromophores by the action of trifluoromethanesulfonic acid.



Scheme 4. Synthesis of the PMB-protected chromophore (**VIII**).

Synthetic approach no. 3. An alternative approach to the synthesis of dehydrotyrosine derivatives, which are the immediate precursors of chromophores, involves the acylation of the amino group of *O*-protected dehydrotyrosine (**IX**) [16] (Scheme 5). However, compound (**IX**) in the neutral state has nucleophilicity too low to be introduced into peptide coupling with activated amino acid derivatives such as acid chlorides, azlactones, and the esters of pentafluorophenol and *N*-hydroxybenzotriazole (R = 2-phenyl-1-aminoethyl). It is known that the amino group of *O*-protected dehydrotyrosine can be deprotonated by a strong base [17]. However, this approach appeared to be inapplicable to *N*-acylamino acids that were not protected at the amide group, probably due to the intramolecular azlactonization in a strongly basic medium.



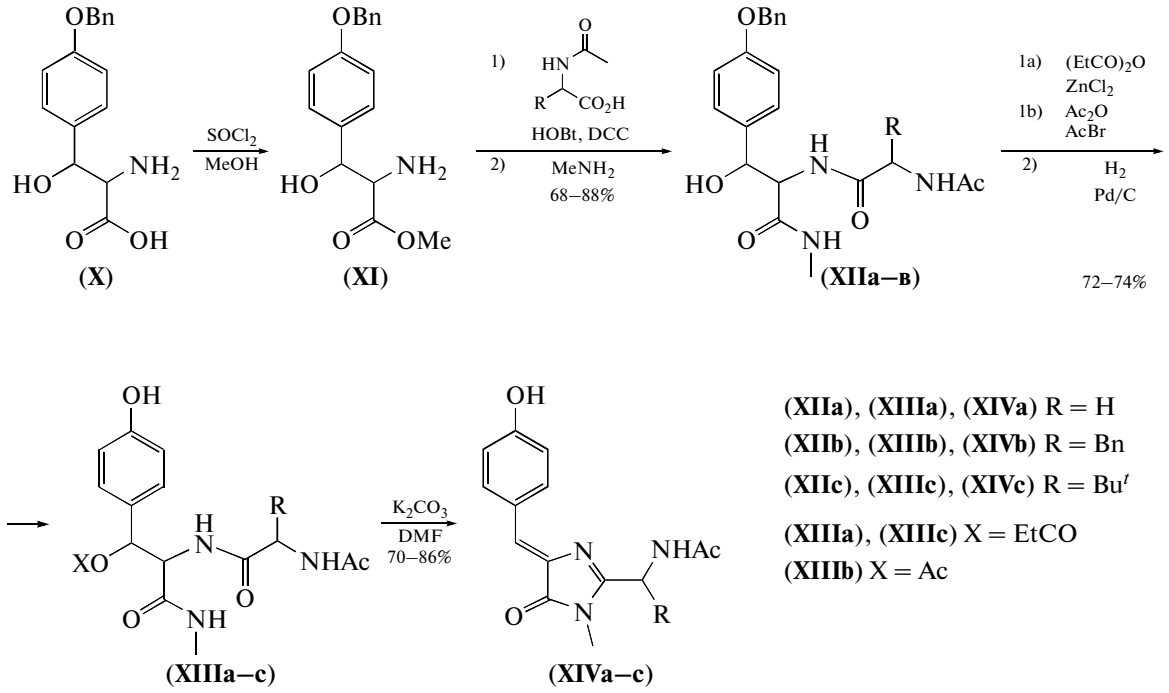
Scheme 5. Synthesis of protected dehydrotyrosine (**IX**) and a study of peptide synthesis with its participation.

Synthetic approach no. 4. We used protected β-hydroxytyrosine (**X**) (Scheme 6) as a synthetic analogue of dehydrotyrosine (**IX**). This compound is easily obtained from glycine and 4-benzyloxybenzaldehyde [18] as a mixture of *rel-(2S,3R)-* and *rel-(2S,3S)* isomers (3.2 : 1), which was subsequently used without separation.

The *N*-acetylation of amine (**XI**) by *N*-acetylamino acids [AcGly, AcPhe, AcLeu^t[2] in the presence of HOBt followed by treatment with aqueous methylamine leads to target dipeptides of β-hydroxytyrosine (**XIIa**)–(**XIIc**) with a good yield.

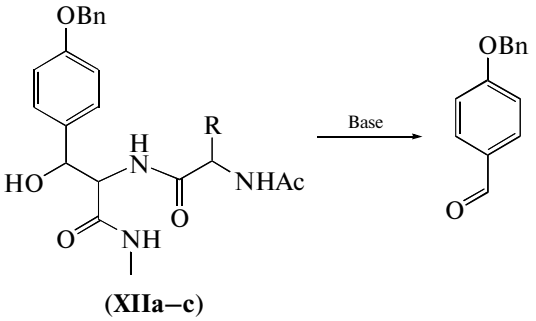
As opposed to the literature data on related compounds [19], the treatment of compounds (**XIIa**)–(**XIIc**) with acetyl chloride or tosyl chloride in the presence of a wide range of bases does not lead to the formation of target acetate or tosylate, as well as the corresponding elimination product. The main reaction product is 4-benzyloxybenzaldehyde (Scheme 7).

[2] Leu^t, *tert*-leucine.



Scheme 6. Synthesis of chromophores (**XIVa**)–(**XIVc**) from β-hydroxytyrosine derivatives.

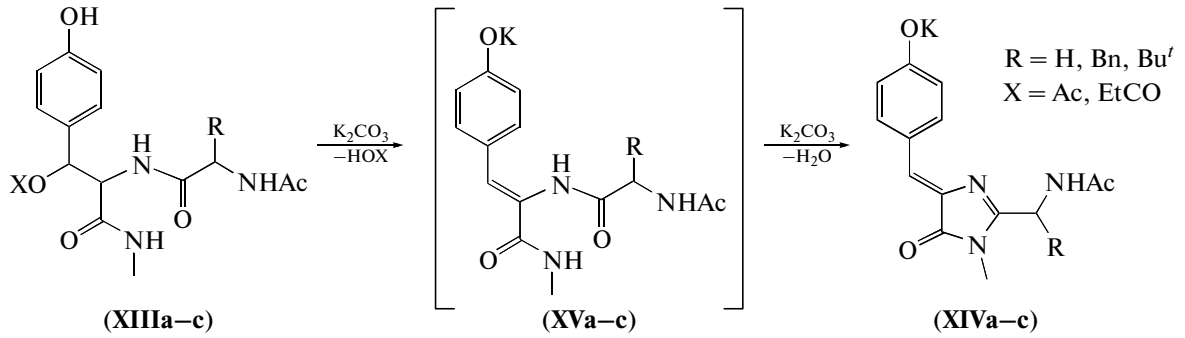
The problem was solved by using stronger Lewis acids. The best yield of acylation products for inactive glycine and *tert*-leucine residues [compounds (**XIIa**) and (**XIIc**)] was with the use of a combination of reagents $ZnCl_2$–$(EtCO)_2O$. In the case of phenylalanine residue (**XIIb**), acetyl bromide in acetic anhydride was applied since, upon heating in the presence of zinc chloride, the corresponding dihydroisoquinoline (a product of the Friedel–Crafts reaction between the acetylamino group and the benzene ring of phenylalanine) formed.



Scheme 7. Base-catalyzed fragmentation of *N*-acylated β-hydroxytyrosine (**XIIa**)–(**XIIc**).

The hydration products (**XIIIa**)–(**XIIIc**) were used in the elimination–cyclization reaction without separating the intermediate products of the carboxylate elimination. The intermediate dehydrotyrosine (**XVc**) formed only in the case of the *tert*-leucine residue (Scheme 8, R = Bu^t, X = EtCO); in this process, the cyclization stage required more time than in the other cases. Presumably, this is related to the sterical effect of the *tert*-butyl group, which hinders the attack of the terminal amide nitrogen at the carbonyl group.

Compared with the acetyl group, the propionyl group (X = EtCO) provides higher yields at the stage of elimination–cyclization of compounds (**XIIIa**)–(**XIIIc**), probably due to a higher resistance to the hydrolysis by water released during the reaction. The addition of molecular sieves (4 Å) makes it possible to increase the yield by 5–10% with the use of acetate. Previously, we have shown the advantage of using the nonnucleophilic cesium carbonate in the cyclization reaction [5]. However, in the case of the hydrophilic glycine chromophore (**XIVa**), cesium carbonate was substituted for by potassium carbonate due to the difficulties in separating the product after the reaction. The elimination–cyclization stage should be carried out in the absence of oxygen since α-acylamino-substituted chromophores in the base medium undergo autooxidation. The glycine-containing chromophore (**XIVa**) undergoes autooxidation to the greatest extent. During its synthesis, the inert conditions should be observed till the stage of the neutralization of the reaction mixture (for more detail, see the Experimental section). It should be noted that chromophores without the α-acylamino group (including 2,3-dimethyl-5-arylidene-3,5-dihydro-4*H*-imidazol-4-one) are prone to autooxidation to a much lesser extent, and the cyclization is usually conducted without any precautions.



(XIIIa–c) (XVa–c) (XIVa–c)

Scheme 8. Mechanism of elimination–cyclization of β-(*O*-acyloxy)tyrosine derivatives (**XIIIa**)–(**XIIIc**).

CONCLUSIONS

Studying the transformations of biomimetic precursor compounds into red chromophores under different conditions and by the action of various reagents will make it possible in future to model processes occurring in natural fluorescent proteins and elucidate the mechanisms of the formation of chromophores.

EXPERIMENTAL

NMR spectra were recorded on a Bruker Avance 700 and a Bruker Avance III 800 spectrometers (Germany, 700 and 800 MHz, respectively; chemical shifts, δ, ppm, spin coupling constant, *J*, Hz). Mass spectrometry analysis was carried out on time-of-flight (TOF) spectrometers ISI RAS MX-5311 (Russia) and Bruker Daltonics UltraFlex TOF/TOF (MALDI) (Germany).

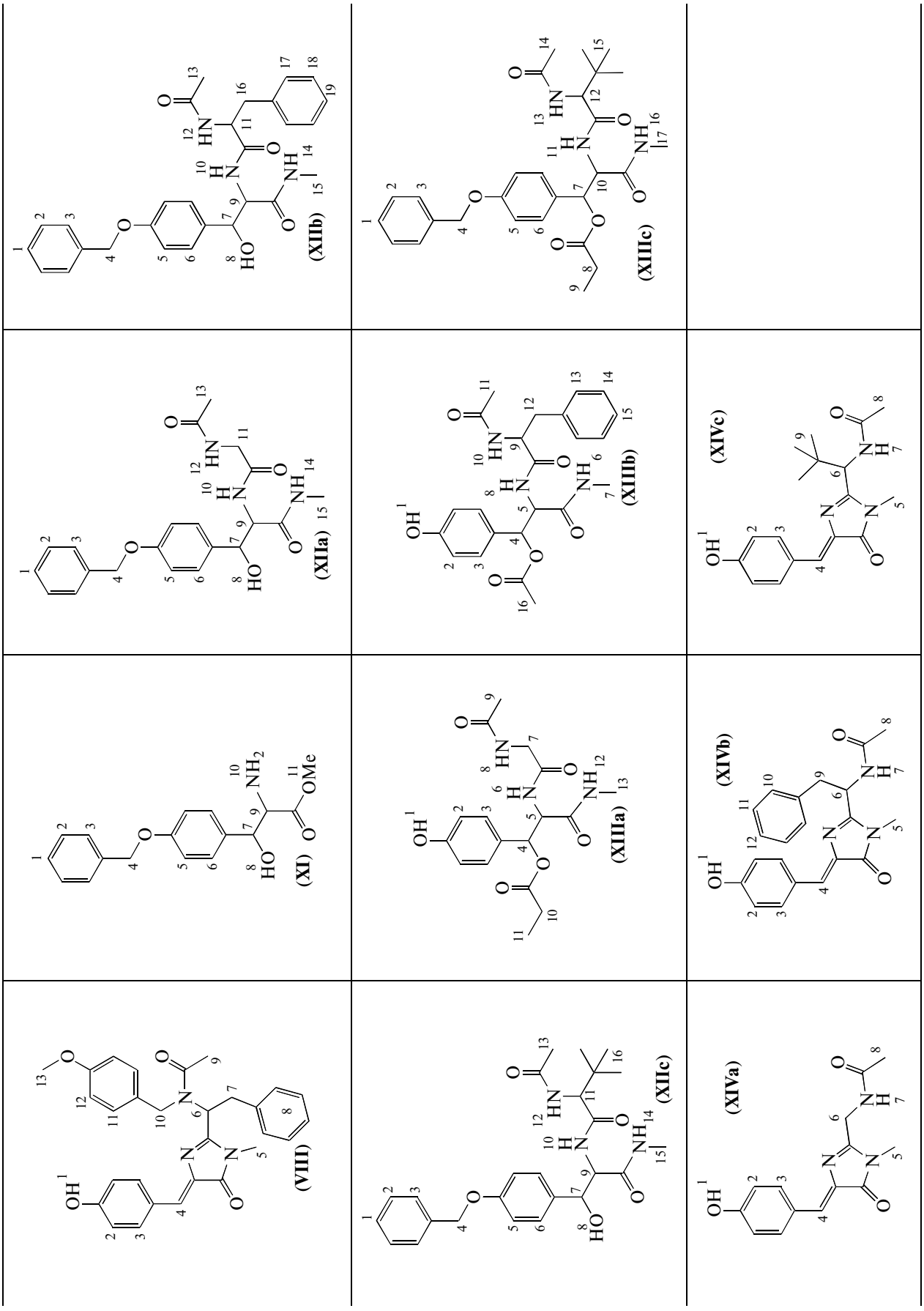
The following compounds were synthesized and characterized by the methods described in the literature: 4-(4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one [3], *N*-acetyl-*tert*-leucine (3,3-dimethyl-2-(*N*-acetylamino)butanoic acid) [20], 4-benzyloxybenzaldehyde [21], ethyl ether of 2-amino-3-(4-(benzyloxy)phenyl)acrylic acid [22], and 2-amino-3-(4-(benzyloxy)phenyl)-3-hydroxypropanoic acid [18].

2-(Bromomethyl)-1-methyl-4-(4-((*tert*-butyldimethylsilyl)oxy)benzylidene)-4,5-dihydro-1*H*-imidazol-5(4*H*)-one (I). Diisopropylethylamine (2.1 mmol, 362 μl) and TBSCl (2.1 mmol, 316 mg) were added to a suspension of 4-(4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one (2.08 mmol, 450 mg) in dichloromethane (20 mL). The mixture was stirred until the complete dissolution of the starting substance and left to stand for 16 h at room temperature. The mixture was diluted with dichloromethane (30 mL), washed with water (20 mL) and a brine (20 mL), and dried over Na_2SO_4. The pale yellow powder obtained after evaporation was purified by column chromatography (hexane–ethyl acetate 3 : 1) to yield 616 mg (89%) as an almost colorless solid substance. NBS (2 mmol, 356 mg) was added to a solution of silylated chromophore (0.90 mmol, 335 mg) in CCl_4 (9 mL), after which AIBN was added over a period of 2.5 h under boiling (in all 40 mg). After the termination of the reaction (control by TLC, hexane–ethyl acetate 3 : 1), the solution was separated and evaporated *in vacuo*. The reaction product was purified by column chromatography (hexane–ethyl acetate 4 : 1). Yield: 210 mg (50%); ^{1}H NMR (800 MHz, $CDCl_3$): 0.25 (6 H, s, CH_3-Si), 1.01 (9 H, s, TBS), 3.32 (3 H, s, N-CH_3), 4.32 (2 H, s, CH_2), 6.91 (2 H, d, *J* 8.5, H_{arom}), 7.24 (1 H, s, H_{vinyl}), 8.08 (2 H, d, *J* 8.5, H_{arom}).

2-(Azidomethyl)-1-methyl-4-(4-((*tert*-butyldimethylsilyl)oxy)benzylidene)-4,5-dihydro-1*H*-imidazol-5(4*H*)-one (II). Sodium azide (0.23 mmol, 15 mg) was added to a solution of compound (**I**) (0.13 mmol, 60 mg) in absolute ethanol (6 mL), and the solution was stirred for 15 min (full conversion by TLC: hexane–ethyl acetate 3 : 1). The reaction mixture was evaporated *in vacuo* at room temperature, and the product was purified by column chromatography (hexane–ethyl acetate 4 : 1). Yield: 47 mg (87%). The resulting azide (**II**) decomposes slowly at room temperature both in the pure form and in solution in EtOAc. With slight heating (at about 50°C), the substance turns dark and melts with the release of gas. ^{1}H NMR (800 MHz, $CDCl_3$): 0.24 (6 H, s, CH_3-Si), 0.99 (12 H, s, TBS), 3.22 (3 H, s, N-CH_3), 3.72 (2 H, s, CH_2), 6.88 (2 H, d, *J* 8.6, H_{arom}), 7.15 (1 H, s, H_{vinyl}), 8.08 (2 H, d, *J* 8.6, H_{arom}).

(2*S*)-2-(*N*-(4-Methoxybenzyl)acetylamino)-3-phenylpropionic acid (VII). Acetic anhydride (18 mmol, 1.70 mL) and triethylamine (25 mmol, 3.50 mL) were added to a suspension of *N*-(4-methoxybenzyl)-*L*-phenylalanine (12 mmol, 3.46 g) in chloroform (40 mL). The mixture was stirred until complete dissolution. After 24 h, the transparent yellow solution was washed with water, evaporated, and redissolved in 1 M NaOH. The resulting solution was washed with ethyl acetate (a colored contamination separated out), acidified by dilute hydrochloric acid to pH 2, and extracted with ethyl acetate. The extract was washed

Numbering of atoms for the assignment of NMR spectra



with a brine, dried over sodium sulfate, and evaporated. The product was recrystallized from dioxane. Yield: 3.0 g (76%); ^{1}H NMR (800 MHz, DMSO-d_6, major rotamer): 1.92 (3 H, s, Ac), 3.02 (1 H, dd, *J* 9.1, 13.9, Phe-CH_2), 3.19 (1 H, dd, *J* 5.9, 13.9, Phe-CH_2), 3.70 (3 H, s, PMB-OMe), 3.88 (1 H, d, *J* 16.4, PMB-CH_2), 4.28 (1 H, dd, *J* 5.9, 9.1, Phe-CH), 4.31 (1 H, d, *J* 16.4, PMB-CH_2), 6.80 (2 H, d, *J* 8.6, PMB-CH), 7.05 (2 H, d, *J* 8.6, PMB-CH), 7.09–7.25 (5 H, m, Phe-arom).

(4*Z*)-2-[(1*R*,*S*)-1-(*N*-Acetyl-*N*-(4-methoxybenzyl)) amino-2-phenylethyl]-3-methyl-4-(4-hydroxybenzylidene)-4,5-dihydro-1*H*-imidazol-5-one (VIII). DCC (3.4 mmol, 700 mg) was added to a solution of *N*-PMB-*N*-acetylphenylalanylglycine (3.31 mmol, 1.27 g) in tetrahydrofuran (15 mL). The mixture was stirred for 15 h and filtered. 4-Acetoxybenzaldehyde (3.31 mmol, 543 mg) and triethylamine (3.4 mmol, 0.48 mL) were added to the resulting solution. After 4 h, 40% aqueous methylamine (17 mmol, 1.43 mL) was added. After additional stirring for 20 min, the reaction mixture was diluted with ethyl acetate (50 mL) and washed with 0.5 M hydrochloric acid, 0.5 M NaOH, and a brine. After evaporation, the pure product (as indicated by TLC; chloroform–ethanol 15 : 1) was recrystallized from alcohol, ethyl acetate. Yield: 315 mg (19%). The resulting dehydrotyrosine (0.608 mmol, 305 mg) was dissolved in aqueous DMF (40 mL), and NaOH (0.67 mmol, 27 mg) was added. Argon was passed through the reaction mixture, after which the mixture was refluxed for 2 h, neutralized by hydrochloric acid, and evaporated *in vacuo*. The residue was dissolved in an ethyl acetate–water mixture. The organic layer was washed with water and a brine and dried over sodium sulfate. After evaporation, the residue was chromatographed in the system chloroform–ethanol 15 : 1. Yield: 150 mg (51%). Along with the major product, brightly colored contaminations were separated in trace amounts, which correspond, according to the MALDI-MS data, to the dehydration product (*m/z* 482), the product of the elimination of the PMB-acetamide group (*m/z* 305), and α-ketone (*m/z* 321). ^{1}H NMR (800 MHz, $CDCl_3$): 2.09 (3 H, s, H9), 2.98 (3 H, s, H5), 3.16 (1 H, dd, *J* 6.1, 13.7, H7), 3.51 (1 H, dd, *J* 8.8, 13.7, H7), 3.74 (3 H, s, H13), 4.65 (1 H, d, *J* 16.9, H10), 4.69 (1 H, d, *J* 16.9, H10), 6.17 (1 H, dd, *J* 6.1, 8.8, H6), 6.76 (2 H, d, *J* 8.6, H12), 6.92 (2 H, d, *J* 8.6, H2), 6.95 (2 H, d, *J* 8.6, H3), 7.01 (1 H, s, H4), 7.19–7.30 (5 H, m, H8), 8.12 (H, d, *J* 8.6, H3); MALDI-MS, *m/z*: 484 ($[M + H]^+$), 506 ($[M + Na]^+$).

Methyl ester of *rel*-(2*S*,3*R*,*S*)-2-amino-3-(4-(benzyloxy)phenyl)-3-hydroxypropionic acid (XI). To a suspension of compound (**X**) (47.3 mmol, 13.6 g) in MeOH (80 mL), $SOCl_2$ (0.1 mol, 7.8 mL) was added dropwise at −10°C under vigorous stirring. The mixture was boiled for 3 h and evaporated *in vacuo*. The solid residue was immersed in 0.5 M HCl (200 mL) and shaken thoroughly with ethyl acetate to remove colored nonpolar contaminations. The aqueous suspension was coated with a layer of ethyl acetate (150 mL) and neutralized by a 10% aqueous solution of Na_2CO_3 (100 mL) under vigorous stirring. The aqueous phase was additionally extracted by ethyl acetate (2 × 60 mL). Combined extracts were washed with a brine, dried over Na_2SO_4, and evaporated. The resulting compound (**XI**) of about 90% purity was additionally purified by column chromatography ($CHCl_3$–EtOH, 10 : 1). Yield: 11.1 g (78%; according to NMR data, a mixture of *rel*-(2*S*,3*R*) and *rel*-(2*S*,3*S*) isomers 3.2 : 1 [18]) in the form of slowly crystallizing oil; mp 119°C (decomposition); *rel*-(2*S*,3*S*) isomer: ^{1}H NMR (800 MHz, DMSO-d_6): 1.8–2.6 (3 H, br, H8 and H10), 3.60 (1 H, d, *J* 4.9, H9), 3.66 (3 H, s, H11), 4.83 (1 H, d, *J* 4.9, H7), 5.06 (2 H, s, H4), 6.96 (2 H, d, *J* 8.6, H5), 7.28 (2 H, d, *J* 8.6, H6), 7.32–7.43 (5 H, m, H1, H2 and H3); *rel*-(2*S*,3*R*) isomer: ^{1}H NMR (800 MHz, DMSO-d_6): 1.8–2.6 (3 H, br, H8 and H10), 3.74 (3 H, s, H11), 4.90 (1 H, d, *J* 6.1, H7), 5.05 (2 H, s, H4), 6.94 (2 H, d, *J* 8.7, H5), 7.20 (2 H, d, *J* 8.7, H6), 7.32–7.43 (5 H, m, H1, H2, and H3).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-(2-acetylaminoacetylamino)-3-(4-(benzyloxy)phenyl)-3-hydroxypropionic acid (XIIa).** A 1 M solution of DCC in tetrahydrofuran (12 mmol, 12 mL) was added at 0°C to a solution of acetylglycine (12 mmol, 1.40 g), ^{i}Pr$_2$NEt (12 mmol, 2.09 mL), and HOBt (12 mmol, 1.62 g) in DMF (30 mL). The mixture was stirred for 1 h at room temperature, a solution of compound (**XI**) (11 mmol, 3.31 g) in DMF (10 mL) was added, and the stirring was continued for 12 h. One mL of AcOH was added (to destroy DCC residues), and the mixture was stirred for an additional 20 min and filtered. DMF was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (120 mL), washed with water, 0.5 M HCl (to remove unreacted amine), a saturated $NaHCO_3$ solution (to remove HOBt and acetylglycine), and a brine, and dried over Na_2SO_4. After the evaporation of ethyl acetate, the resulting methyl ester (pure, as indicated by TLC in EtOAc–EtOH 2 : 1) was dissolved in MeCN (60 mL). Aqueous methylamine (60 mmol, 5.17 mL) was added to the solution, and the mixture was allowed to stand at 40°C until full conversion according to TLC (about 3 h). A colorless fine-crystalline precipitate occurs, which, after washing with acetonitrile and ester, represents a spectrally pure compound (**XIIa**) (3.03 g, yield for two stages 69%). After evaporation from mother liquor and column chromatography (EtOAc–EtOH 3 : 1), 0.76 g (19%) of compound (XIIa) was additionally obtained. The total yield for two stages was 3.79 g (88%, a mixture of stereoisomers); mp 191°C; ^{1}H NMR (800 MHz, DMSO-d_6, major isomer/rotamer): 1.84 (3 H, s, H13), 2.58 (3 H, d, *J* 4.7, H15), 3.60 (1 H, dd, *J* 5.6, 16.4, H11), 3.77 (1 H, dd, *J* 5.6, 16.4, H11), 4.27 (1 H, dd, *J* 3.2, 8.8, H9), 5.02 (1 H, t, *J* 3.2, 4.6, H7), 5.06 (2 H, s, H4), 5.60 (1 H, d, *J* 4.6, H8), 6.91 (2 H, d,

J 8.6, H5), 7.25 (2 H, d, *J* 8.6, H6), 7.31–7.44 (5 H, 3m, H1, H2, H3), 7.67 (1 H, q, *J* 4.6, H14), 7.74 (1 H, d, *J* 8.8, H10), 8.07 (1 H, t, *J* 5.6, H12); ESI-MS, *m/z*: 400 ($[M + H]^+$), 382 ($[M - H_2O + H]^+$), 422 ($[M + Na]^+$).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-((2*R*,*S*)-2-acetylamino(benzyl)acetylamino)-3-(4-(benzyloxy) phenyl)-3-hydroxypropionic acid (XIIb).** Compound (**XIIb**) was obtained in the same way as derivative (**XIIa**). The final product was purified by column chromatography ($CHCl_3$–EtOH 85 : 15). Yield for two stages 68% (a mixture of stereoisomers); ^{1}H NMR (700 MHz, DMSO-d_6, major isomer/rotamer): 1.72 (3 H, s, H13), 2.56 (3 H, d, *J* 4.7, H15), 2.66 (1 H, dd, *J* 10.3 and 13.9, H16), 2.87 (1 H, dd, *J* 4.9, 13.9, H16), 4.24 (1 H, dd, *J* 2.3, 8.6, H9), 4.59 (1 H, m, H11), 5.05 (3 H, m, H4 and H7), 5.61 (1 H, d, *J* 4.4, H8), 6.89 (2 H, d, *J* 8.8, H5), 7.05 (1 H, d, *J* 7.1, H), 7.18–7.41 (5 H, m, H1, H2, and H3), 7.22 (2 H, d, *J* 8.8, H6), 7.53 (1 H, q, *J* 4.7, H14), 7.65 (1 H, d, *J* 8.6, H10), 8.09 (1 H, d, *J* 8.3, H12).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-((2*R*,*S*)-2-acetylamino(*tert*-butyl)acetylamino)-3-(4-(benzyloxy) phenyl)-3-hydroxypropionic acid (XIIc).** Compound (**XIIc**) was obtained in the same way as derivative (**XIIa**), except that the reaction mixture was stirred for 2 h before, and for 24 h after, the addition of amine (**XI**). The final product was purified by column chromatography ($CHCl_3$–EtOH 10 : 1). Yield for two stages 73% (a mixture of stereoisomers); mp 156°C; ^{1}H NMR (700 MHz, $CDCl_3$, major isomer/rotamer): 0.67 (9 H, s, H16), 1.89 (3 H, s, H13), 2.58 (3 H, d, *J* 4.6, H15), 4.05 (1 H, d, *J* 6.8, H11), 4.30 (1 H, dd, *J* 2.9, 8.8, H9), 5.07 (2 H, m, H4), 5.16 (1 H, dd, *J* 2.9, 5.3, H7), 5.51 (1 H, d, *J* 5.3, H8), 6.87 (2 H, d, *J* 8.6, H5), 7.21 (1 H, q, *J* 4.6, H14), 7.27 (2 H, d, *J* 8.6, H6), 7.30–7.43 (5 H, m, H1, H2, H3), 7.73 (1 H, d, *J* 6.8, H12), 7.87 (1 H, d, *J* 8.8, H10); ESI-MS, *m/z*: 456 ($[M + H]^+$), 438 ($[M - H_2O + H]^+$), 478 ($[M + Na]^+$).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-(2-acetylaminoacetylamino)-3-(4-(hydroxyphenyl)-3-(propionyloxy) propionic acid (XIIIa).** A mixture of compound (**XIIa**) (3.45 mmol, 1.38 g), propionic anhydride (15 mL), and $ZnCl_2$ (1 mmol, 136 mg) was stirred at 90°C until the complete dissolution of the starting compound (**XIIa**) and full conversion according to TLC (total time 4 h). An excess of propionic anhydride and propionic acid was evaporated *in vacuo* at 70°C. The residue was reevaporated twice with isopropanol, dissolved in an ethanol–chloroform mixture, and passed through a silica gel layer. After the evaporation of solvents, the solid colorless residue was recrystallized from isopropanol. To a suspension of the recrystallization product in methanol, 10% Pd/C (200 mg) and TFA (50 μl) were added, and the resulting mixture was hydrated at atmospheric pressure for 3 h (100% conversion according to TLC). The solution was filtered through a paper filter and evaporated. The product was purified by column chromatography ($CHCl_3$–EtOH 85 : 15). Yield for two stages 0.91 g (72%); ^{1}H NMR (700 MHz, D_2O, major isomer/rotamer): 1.24 (3 H, t, *J* 7.13, H11), 2.18 (3 H, s, H9), 3.08 (3 H, s, H13), 3.71 (2 H, q, *J* 7.13, H10), 4.34 (2 H, s, H7), 6.92 (2 H, d, *J* 8.7, H2), 6.95 (1 H, s, H4), 7.86 (2 H, d, *J* 8.7, H3); ESI-MS, *m/z*: 366 ($[M + H]^+$), 388 ($[M + Na]^+$), 292 ($[M - C_2H_5CO_2H + H]^+$).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-((2*R*,*S*)-2-acetylamino(benzyl)acetylamino)-3-(4-(hydroxyphenyl)-3-(acetoxy)propionic acid (XIIIb).** A solution of AcBr in Ac_2O (2.0 mmol, 0.15 mL in 1.5 mL of Ac_2O) was added to a solution of compound (**XIIb**) (2.0 mmol, 1.0 g) in methylene chloride (10 mL). After 1 h (100% conversion by TLC, chloroform–ethanol 9 : 1), the reaction mixture was diluted with ethyl acetate (50 mL) and washed with saturated $NaHCO_3$ solution and a brine. After evaporation, the residue was dried at 80°C using an oil pump. The acylation product was suspended in methanol (30 mL), Pd/C (150 mg) and trifluoroacetic acid (100 μl) were added, and the resulting mixture was hydrated at room temperature and atmospheric pressure (TLC, chloroform–ethanol 7 : 1). The solution was filtered through a paper filter and evaporated. The product was purified by column chromatography ($CHCl_3$–EtOH 8 : 1). Yield for two stages 0.65 g (74%); ^{1}H NMR (800 MHz, DMSO-d_6, major isomer/rotamer): 1.91 and 2.05 (6 H, 2s, H11 and H16), 2.63 (3 H, d, *J* 4.9, H7), 2.85 and 3.00 (2 H, 2 m, H12), 4.63 (m, H9), 4.74 (1 H, dd, *J* 6.1, 8.8, H5), 6.15 (1 H, d, *J* 6.1, H4), 6.71–7.26 (8 H, m, H3, H10, H13, H14, and H15), 6.74 (2 H, d, *J* 8.6, H2), 6.84 (1 H, q, *J* 4.9, H6), 7.09 (1 H, d, *J* 8.8, H8).

***N*-Methylamide of *rel*-(2*S*,3*R*,*S*)-2-((2*R*,*S*)-2-acetylamino(*tert*-butyl)acetylamino)-3-(4-hydroxyphenyl)-3-(propionyloxy)propionic acid (XIIIc).** This compound was prepared by the above-described scheme. Yield: 81%; ^{1}H NMR (700 MHz, $CDCl_3$, major isomer/rotamer): 0.86 (9 H, s, H15), 0.98 (3 H, t, *J* 7.5, H9), 1.87 (3 H, s, H14), 2.27 (2 H, dq, *J* 2.4, 7.5, H8), 2.42 (3 H, d, *J* 4.7, H17), 4.33 (1 H, d, *J* 9.5, H12), 4.70 (1 H, dd, *J* 6.9, 8.8, H10), 5.06 (2 H, s, H4), 6.85 (1 H, d, *J* 6.9, H7), 6.92 (2 H, d, *J* 8.8, H5), 7.19 (2 H, d, *J* 8.8, H6), 7.33–7.43 (5 H, m, H1, H2, H3), 7.73 (1 H, d, *J* 9.5, H13), 7.78 (1 H, q, *J* 4.7, H16), 8.07 (1 H, d, *J* 8.8, H11); ESI-MS, *m/z*: 512 ($[M + H]^+$), 438 ($[M - C_2H_5CO_2H + H]^+$), 534 ($[M + Na]^+$).

(4*Z*)-2-(*N*-Acetylamino)methyl-3-methyl-4-(4-hydroxybenzylidene)-4,5-dihydro-1*H*-imidazol-5-one (XIVa). Compound (**XIIIa**) (0.60 mmol, 220 mg), K_2CO_3 (1.28 mmol, 176 mg), MS 4 Å, and DMF (20 mL) were placed in a flask equipped with a septum and magnetic stirrer. Argon was blown through the mixture for 20 min, and then the mixture was stirred at 110°C for 35 min. After cooling to room temperature, a solution of NH_4Cl (1.5 mmol, 80 mg) in H_2O (5 mL) was added. The resulting orange solution was evapo-

rated *in vacuo* at 60°C. The residue was reevaporated with ethanol and a threefold (relative to the solid substance) volume of silica gel. The resulting powder was chromatographed on a column of silica gel in the system EtOAc–EtOH 20 : 1–15 : 1. Yield: 121 mg (74%, a thermodynamic mixture of *Z/E* isomers 10 : 1); ^{1}H NMR (800 MHz, DMSO-d_6, *Z* isomer): 1.94 (3 H, s, H8), 3.08 (3 H, s, H5), 4.32 (2 H, d, *J* 5.6, H6), 6.85 (2 H, d, *J* 8.8, H2), 6.99 (1 H, s, H4), 8.11 (2 H, d, *J* 8.8, H3), 8.41 (1 H, t, *J* 5.6, H7), 10.16 (1 H, br, H1); ESI-MS, *m/z*: 274 ($[M + H]^+$), 296 ($[M + Na]^+$), 312 ($[M + K]^+$).

(4*Z*)-2-[(1*R*,*S*)-(1-Acetylamino-2-phenyl)ethyl]-3-methyl-4-(4-hydroxybenzylidene)-4,5-dihydro-1*H*-imidazol-5-one (XIVb). Compound (**XIVb**) was synthesized in the same way as derivative (**XIVa**). Yield 70%; ^{1}H NMR (700 MHz, DMSO-d_6, *Z* isomer): 1.78 (3 H, s, H8), 2.97 (3 H, s, H5), 3.07 (1 H, dd, *J* 8.6, 13.7, H9), 3.29 (1 H, dd, *J* 6.4, 13.9, H9), 5.09 (1 H, dd, *J* 6.4, 8.3, and 8.6, H6), 6.86 (2 H, d, *J* 8.8, H2), 7.00 (1 H, s, H4), 7.19–7.31 (5 H, m, H10, H11, and H12), 8.13 (2 H, d, *J* 8.8, H3), 8.53 (1 H, d, *J* 8.6, H7), 10.17 (1 H, s, H1); HSQC-^{1}H-^{13}C (800 MHz, DMSO-d_6, *Z* isomer): 22.7 (H8), 26.8 (H5), 37.7 (H9), 48.0 (H6), 116.1 (H2), 126.6 (H12), 127.8 (H4), 128.4, and 129.7 (H11 and H12), 135.0 (H3).

(4*Z*)-2-[(1*R*,*S*)-(1-Acetylamino-2-dimethyl)propyl]-3-methyl-4-(4-hydroxybenzylidene)-4,5-dihydro-1*H*-imidazol-5-one (XIVc). Compound (**XIVc**) was synthesized in the same way as derivative (**XIVa**). The reaction temperature was maintained at 140°C, and the reaction occurred in two stages according to TLC (the product of the first stage is the substance easily oxidizable by permanganate with a strong absorption in the UV region, which is presumably the product of splitting off of propionate). Yield: 86%; ^{1}H NMR (700 MHz, $CDCl_3$, *Z* isomer): 1.10 (9 H, s, H9) 2.08 (3 H, s, H8), 3.26 (3 H, s, H5), 4.94 (1 H, d, *J* 9.25, H6), 6.66 (1 H, d, *J* 9.25, H7), 6.86 (2 H, d, *J* 8.6, H2), 7.12 (1 H, s, H4), 7.99 (2 H, *J* 8.6, H3), 8.26 (1 H, br s, H1). ESI-MS, *m/z*: 330 ($[M + H]^+$), 352 ($[M + Na]^+$), 368 ($[M + K]^+$).

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